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Specific inhibition of HCN channels slows rhythm differently in atria, ventricle and outflow tract and stabilizes conduction in the anoxic-reoxygenated embryonic heart model

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ABSTRACT

The hyperpolarization-activated cyclic nucleotide-gated (HCN) channels are expressed in pacemaker cells very early during cardiogenesis. This work aimed at determining to what extent these channels are implicated in the electromechanical disturbances induced by a transient oxygen lack which may occur in utero.

Spontaneously beating hearts or isolated ventricles and outflow tracts dissected from 4-day-old chick embryos were exposed to a selective inhibitor of HCN channels (ivabradine 0.1–10 μM) to establish a dose–response relationship. The effects of ivabradine on electrocardiogram, excitation–contraction coupling and contractility of hearts submitted to anoxia (30 min) and reoxygenation (60 min) were also determined. The distribution of the predominant channel isoform, HCN4, was established in atria, ventricle and outflow tract by immunoblotting.

Intrinsic beating rate of atria, ventricle and outflow tract was 164 ± 22 (n = 10), 78 ± 24 (n = 8) and 40 ± 12 bpm (n = 23, mean ± SD), respectively. In the whole heart, ivabradine (0.3 μM) slowed the firing rate of atria by 16% and stabilized PR interval. These effects persisted throughout anoxia-reoxygenation, whereas the variations of QT duration, excitation–contraction coupling and contractility, as well as the types and duration of arrhythmias were not altered. Ivabradine (10 μM) reduced the intrinsic rate of atria and isolated ventricle by 27% and 52%, respectively, whereas it abolished activity of the isolated outflow tract. Protein expression of HCN4 channels was higher in atria and ventricle than in the outflow tract.

Thus, HCN channels are specifically distributed and control finely atrial, ventricular and outflow tract pacemakers as well as conduction in the embryonic heart under normoxia and throughout anoxia-reoxygenation.

1. Introduction

Hyperpolarization-activated cyclic nucleotide-gated (HCN) channels are expressed in nodal, atrial and ventricular tissues, carry the pacemaker funny current (*If*) and control the rate of diastolic depolarization of the sinoatrial node in the adult heart [1,2]. Selective inhibition of these channels slows the cardiac rate [3] without any effect on contractility [4] and can protect against ischemia-induced myocardial injury [5–7]. HCN channels, including the predominant HCN4 isoform, are expressed very early during cardiogenesis and are required for pacemaker activity [8–10]. Pacemaker locus differentiates in the posterior segment of the primitive tubular heart [11,12] and strong *If* current can be generated by hyperpolarization in ventricular cardiomyocytes from the chick (3-day-old) and the mouse (9.5-day-old) embryo [13–15], in contrast to adult ventricular myocytes. Furthermore, hierarchy of intrinsic rhythmicity within the embryonic heart is illustrated by the fact that dissected atria, ventricle and outflow tract beat spontaneously and regularly at their respective firing rate, i.e. atria > ventricle > outflow tract [16]. This observation suggests that membrane density and/or properties of the HCN channels, besides non-HCN pacing mechanisms, might vary from one cardiac region to another.

Throughout the embryonic and fetal life, occasional transient or prolonged reduction in oxygen availability can lead to reversible or irreversible cardiac arrhythmias, threatening fetal growth. The mechanisms underlying hypoxia-induced rhythm abnormalities in the developing heart remain poorly understood and deserve to be investigated. We have previously shown that anoxia and reoxygenation induce rapid and severe electrical and mechanical disturbances in the 4–5-day-old embryonic chick heart model [16,17]. Arrhythmic activity of isolated atria persists throughout anoxia and upon reoxygenation, whereas activity of isolated ventricles rapidly ceases under anoxia and resumes late after reoxygenation. Whether HCN channels are involved in such disturbances

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and whether their inhibition has beneficial consequences, as in the adult heart, remains to be explored.

This study mainly aimed at examining the contribution of the HCN channels to pacemaker activity and conduction in the embryonic heart as well as investigating their role in cardiac dysfunction induced by anoxia and reoxygenation.

2. Material and methods

2.1. Reagents

Standard chemicals were purchased from Sigma (Sigma Aldrich, Buchs, Switzerland). The specific inhibitor of HCN channels ivabradine was kindly provided by Servier (France).

2.2. Preparation and in vitro mounting of the heart

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 [18]). Hearts were carefully excised from embryos and dissected in order to isolate ventricles and outflow tracts. Spontaneously beating hearts or the isolated parts were placed in the culture compartment of a stainless steel airtight chamber. Such device was equipped with two windows for observation as well as measurements and maintained under controlled conditions on the thermostabilized stage (37.5 °C) of an inverted microscope (IMT2 Olympus, Tokyo, Japan) as previously described in detail [17]. Briefly, the culture compartment (300 μl) was separated from the gas compartment by a 15 μm transparent and gas-permeable silicone membrane (RTV 141, Rhône-Poulenc, Lyon, France). Hearts, ventricles and outflow tracts were slightly flattened by the silicone membrane and the resulting thickness of the myocardial tissue facing the gas compartment was approximately 300 μm. Thus, pO_2 at the tissue level could be strictly controlled and rapidly modified (within less than 5 s) by flushing high-grade gas of selected composition through the gas compartment. At this developmental stage, the heart lacks coronary vascularization and the myocardial oxygen requirement is met exclusively by diffusion.

The HCO_3/CO_2 buffered medium was composed of (in mM): 99.25 NaCl; 0.3 NaH_2PO_4; 10 $NaHCO_3$; 4 KCl; 0.79 $MgCl_2$; 0.75 $CaCl_2$; 8 D(+)glucose. This culture medium was equilibrated in the chamber with 2.31% CO_2 in air (normoxia and reoxygenation) or in N_2 (anoxia) yielding a pH of 7.4. Ivabradine was dissolved in the culture medium just before experiments.

2.3. Recording of electrical and contractile activity

Electrical and contractile activities were recorded simultaneously and continuously throughout in vitro experiments as previously described [16].

2.3.1. Electrical activity

ECG recordings of the spontaneously contracting intact hearts were performed using two Ag/AgCl electrodes 1.2 mm apart (diameter 0.625 mm) inserted into the window facing the culture compartment. The atrial and ventricular regions of the heart were placed in the immediate vicinity of these electrodes, which were connected to a differential preamplifier (gain of 2000), resulting in an output signal of 1–5 V peak to peak. This signal was digitized and processed using a powerful data acquisition (IOX, sampling rate: 2 kHz) and analysis system (ECG-Auto) developed by EMKA Technologies (France). ECG displayed characteristic P, QRS and T components, which allowed to assess the beating rate from RR interval (beats/min, bpm), PR interval (ms) and QT duration (ms). Corrected QT (QTc) was calculated using Bazett's formula ($QT/RR^{1/2}$).

2.3.2. Contractile activity

Adjustable phototransistors were positioned over the projected image of the investigated regions allowing detection of edge motion of the myocardial wall, i.e. at the level of atrial pacemaker and ventricular apex of the intact heart. Simultaneously with ECG, myocardial shortening was sampled at a rate of 1 kHz using the same acquisition/analysis system as described above. The actual ventricular shortening at the apex (μm) was determined using video recordings performed just before anoxia and at the end of reoxygenation.

2.3.3. Excitation–contraction coupling

The electromechanical delay (EMD, ms), reflecting the efficiency of excitation–contraction (E–C) coupling, was determined at the level of the right atrium and at the apex of the ventricle by measuring the delay between the very initial phase of the P and QRS components and the initiation of contraction in atrium and ventricle, respectively, as previously described [16].

2.4. Experimental protocols

2.4.1. Dose–response curve

Whole hearts, isolated ventricles and outflow tracts were pretreated with ivabradine at room temperature during 30 min, mounted in the culture chamber and exposed to normoxia during 1 h in the absence (control) or the presence of ivabradine. The tested concentrations of ivabradine ranged from 0.1 to 10 μM. The spontaneous beating rate was determined at the end of the protocol.

2.4.2. Anoxia-reoxygenation protocol

Whole hearts (pretreated or not with 0.3 μM ivabradine) were placed in the chamber, stabilized during 45 min under normoxia and submitted to anoxia (30 min) followed by reoxygenation (60 min). Ivabradine (0.3 μM) was present throughout anoxia-reoxygenation and electrical (ECG) as well as contractile activities were continuously recorded.

2.5. Western Blotting of HCN4 channels

A total of 84 hearts have been used and carefully dissected into atria, ventricle and outflow tract. Six atria, 3 ventricles and 6 outflow tracts were pooled for each sample preparation. Protein extracts were generated from the dissected regions using a lysis buffer (in mM: 20 Tris-acetate (pH 7), 270 sucrose, 1 EGTA, 1 EDTA, 50 NaF, 10 β-glycerophosphate, 10 dithiothreitol (DTT), 10 4-nitrophenyl phosphate disodium salt hexahydrate (PNPP), 1% Triton X-100 and inhibitors of proteases). Insoluble material was removed by a 5 min centrifugation at 10,000 g. Protein concentration was determined by the Bradford method. Samples were denatured in sample buffer (6 min, 95 °C), 20 μg of protein were loaded per lane, separated on 10% SDS-polyacrylamide gels, and transferred to nitrocellulose membranes. Membranes were blocked (overnight at 4 °C) with 5% non-fat milk in PBS and probed (2 h at room temperature) with rabbit polyclonal antibody against HCN4 1:200 (Alomone Labs, Jerusalem, Israel) or GAPDH 1:1000 (Abcam, Cambridge, UK). A control fusion protein was used as control antigen. Anti-HCN4 antibody was preincubated with the antigen (for negative control) into PBS–5% non-fat dry milk for 1 h. After 4 washes in 0.1% Tween 20 PBS the membranes were incubated in 1:10,000 dilution of horseradish peroxidase-conjugated anti-rabbit IgG (Amersham Biosciences) in PBS containing 5% non-fat milk (1 h, room temperature) and then washed 4 times.

Immunoreactive bands were detected with enhanced chemiluminescent procedure using ECL Western Blotting Analysis System (Amersham Biosciences). Autoradiograms were scanned and densitometric analysis performed with Quantity One software (Biorad). Protein bands were normalized using GAPDH in the same sample as reference.

2.6. Statistical analysis

All values are reported as mean ± standard error of the mean (SEM) unless otherwise indicated. The significance of any difference between two groups was assessed with Student *t*-test or repeated measures ANOVA (to compare rates of recovery), while differences in the dose–response of ivabradine were determined using one-way ANOVA completed by Tukey's post hoc test using SPSS software. Mann–Whitney test was used to compare slopes of the PR–RR relationship. The statistical significance was defined by a value of $p < 0.05$.

3. Results

3.1. HCN4 channels are differentially expressed within the embryonic chick heart

Immunoblotting shows that expression of the predominant isoform HCN4 was higher in atria and ventricle than in the outflow tract of the embryonic chick heart (Fig. 1). The specificity of the anti-HCN4 antibody was controlled by immunoblotting with the anti-HCN4 antibody preincubated with the corresponding antigen. The band normally found at about 160 kDa, corresponding to HCN4, disappeared completely in the presence of the antigen (not shown).

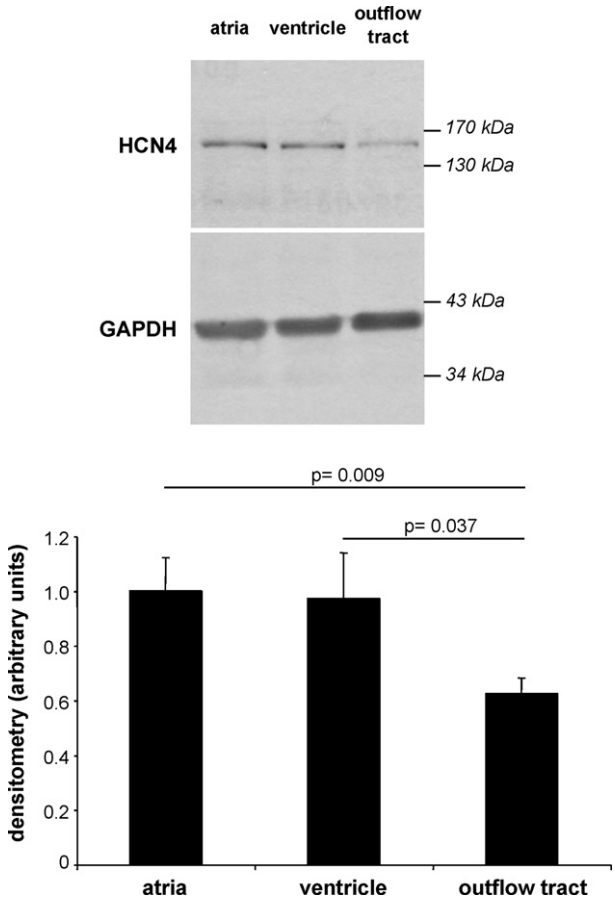

Fig. 1. Representative immunoblot of the HCN4 channel and GAPDH (*upper panel*) and quantitative densitometry of HCN4 corrected for GAPDH (*lower panel*) showing that HCN4 was inhomogeneously distributed in the embryonic chick heart at stage 24HH. Bars represent the mean ± SEM from 14, 12 and 11 independent determinations for atria, ventricle and outflow tract, respectively.

3.2. Dose–response curve for ivabradine was different in atria, ventricle and outflow tract

In vitro, all atria (in whole hearts) and dissected ventricles beat at their intrinsic rate, whereas only about 25% of the dissected outflow tracts beat spontaneously and regularly. Under normoxia, the intrinsic beating rate of the untreated atria, isolated ventricles and outflow tract was 164 ± 22 ($n = 10$), 78 ± 24 ($n = 8$) and 40 ± 12 bpm ($n = 23$, mean ± SD), respectively, and remained stable in vitro. The dose–response curves (Fig. 2) show that inhibition of HCN channels by 1 μM ivabradine significantly ($p < 0.01$) slowed the firing rate of atria (dropping to 134 ± 17 bpm, $n = 6$) and ventricle (dropping to 39 ± 19 bpm, $n = 7$) respectively. The highest concentration of ivabradine (10 μM) further decreased moderately atrial rate, had no additional effect on ventricular rhythm but completely stopped spontaneous activity of the outflow tract (Fig. 2). Thus, the maximal inhibitory effect of ivabradine is achieved at 0.3–1 and 10 μM in ventricle and outflow tract, respectively. Since the ventricular rate was already altered at a low concentration of ivabradine (0.3 μM), we investigated the functional consequences of HCN inhibition during anoxia-reoxygenation at this concentration only.

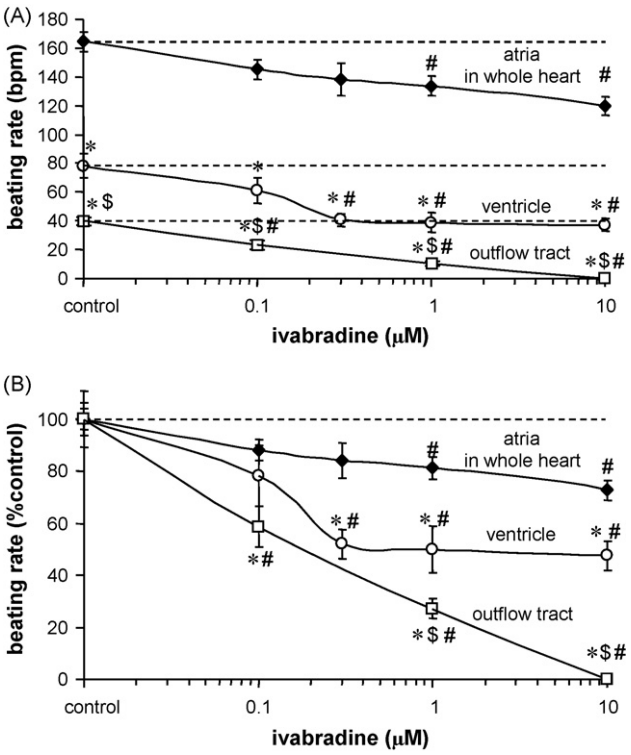

Fig. 2. Dose–response curves of whole hearts (black diamonds), dissected ventricles (open circles) and outflow tracts (open squares) treated with ivabradine under normoxia. The spontaneous beating rate of atria, isolated ventricles and outflow tracts was expressed as raw data (A) or as percent of the respective control value representing ivabradine efficiency (B). Control: untreated preparation; broken lines indicate the control level; bpm: beats per min.; mean ± SEM; $n = 4$–11 for atria and ventricle; $n = 9$–23 for outflow tract; *: $p < 0.01$ dissected ventricle or outflow tract versus atria in whole heart; $: $p < 0.01$ outflow tract versus ventricle; #: $p < 0.01$ versus respective control.

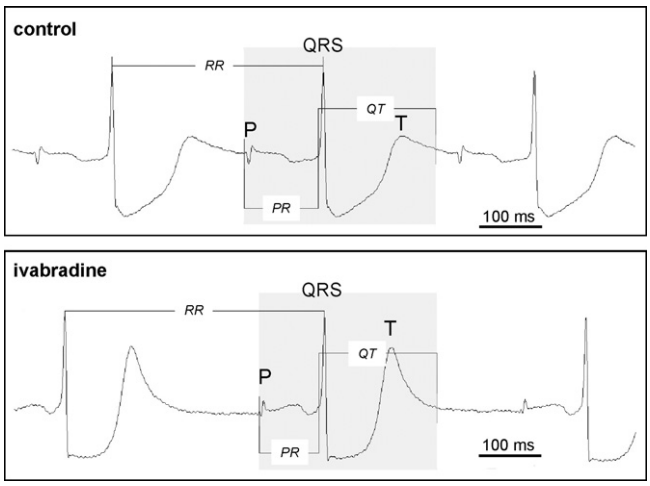

Fig. 3. Representative ECGs illustrating the P, QRS and T components of the embryonic chick heart spontaneously beating in vitro under normoxia and showing how RR, PR and QT intervals have been analysed in control and ivabradine (0.3 μM) treated hearts.

3.3. Inhibition of HCN channels had a bradycardic effect associated with a stabilization of conduction without affecting the pattern of arrhythmias throughout anoxia-reoxygenation

Under preanoxic conditions, ivabradine (0.3 μM) reduced atrial rate and PR interval by 20%, but did not alter QT duration, ventricular EMD and ventricular shortening (Figs. 3 and 4). It should be noticed that the preanoxic atrial rate of untreated hearts (186 ± 9 bpm, $n = 5$, Fig. 4) was slightly higher than heart rate of the controls of the dose–response experiments (164 ± 7 bpm, $n = 10$, Fig. 2) although it did not reach statistical significance. Such a difference between the two series of experiments, combined with a higher coefficient of variability of atrial rate at 0.3 μM ivabradine in Fig. 2 (18%; $n = 5$) than that in Fig. 4 (12%; $n = 5$), could explain that the bradycardic effect of 0.3 μM ivabradine on atria was significant during preanoxia ($p = 0.03$) but not under the conditions of the dose–response determination ($p = 0.09$). As we recently described [16], anoxia induced bradycardia, atrial ectopy, 1st, 2nd and 3rd degree A-V blocks as well as transient arrests followed by bursting activity. Reoxygenation triggered also Wenckebach phenomenon and ventricular escape beats. All hearts fully recovered after 30–50 min of reoxygenation. Relative to control after 10 min anoxia, ivabradine augmented the mean atrial rate measured during bursts of activity with no effect on other functional parameters (Fig. 4). During reoxygenation, ivabradine shortened PR interval with respect to untreated hearts. Nevertheless, the types, incidence and duration of arrhythmias were not significantly affected by ivabradine (Table 1). Relative to control, ivabradine did not alter QTc neither under normoxia (0.25 ± 0.02 and 0.28 ± 0.03 s, in control and treated group, respectively, $n = 5$) nor throughout reoxygenation (peaking after 9 min of reoxygenation at 0.35 ± 0.07 and 0.30 ± 0.02 s in control and ivabradine, respectively). However, the mean of all the QTcs which were possible to determine, i.e. in the absence of AV blocks, at 11, 13, 20, 25 and 30 min of anoxia (see Fig. 4), was longer in the ivabradine group (0.28 ± 0.06 s, $n = 17$) than in the control group (0.23 ± 0.04 s, $n = 16$; ± SD) ($p = 0.006$). Furthermore, as previously described [19], atrial EMD was longer than ventricular EMD under baseline condition (i.e. 19.9 ± 0.9 ms vs 12.1 ± 1.8 ms) and was not significantly affected by reoxygenation and ivabradine (not shown).

Table 1
Inhibition of HCN channels by ivabradine (0.3 μM) did not significantly influence the pattern of arrhythmias in the embryonic heart during anoxia and reoxygenation; time is given in minutes; mean ± SD; $n = 5$ for each condition.

	Anoxia				Reoxygenation				
	First atrial arrest	First ventricular arrest	Duration of AV blocks	Duration of cardioplegia	Atrial resumption	Ventricular resumption	Duration of AV blocks	Duration of Wenckebach	End of arrhythmias
Control	12.3 ± 10.9	3.4 ± 2.0	11.8 ± 8.9	7.5 ± 9.3	1.5 ± 1.6	3.0 ± 2.2	11.9 ± 9.7	14.7 ± 17.1	33 ± 15
Ivabradine	3.0 ± 1.4	2.7 ± 1.3	6.4 ± 4.8	15.8 ± 3.2	0.8 ± 0.6	1.4 ± 0.8	10.7 ± 5.0	8.0 ± 11.6	25 ± 9

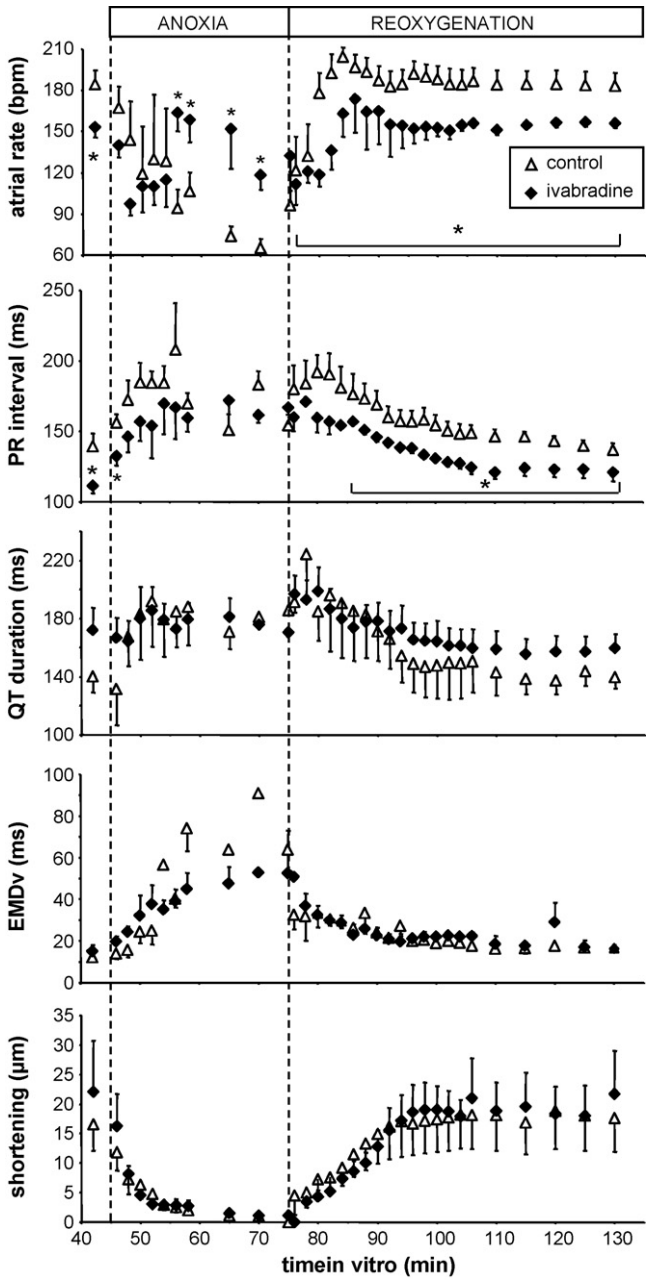

Fig. 4. In the whole heart, ivabradine decreased atrial rate and shortened PR interval under normoxia (preanoxia) and throughout reoxygenation. By contrast, QT duration, ventricular electromechanical delay (EMDv) and ventricular shortening were not affected. Mean ± SEM; $n = 5$ for each condition; *: $p < 0.05$ versus control.

3.4. Correlation between heart rate and atrio-ventricular conduction

Fig. 5 clearly shows that atrio-ventricular conduction under normoxia depended on beating rate since the longer the RR interval, the shorter the PR interval, regardless if ivabradine was applied or not. Furthermore, a beat-to-beat analysis performed in individual hearts revealed that specific inhibition of HCN channels by ivabradine stabilized conduction, specially when RR varied between 370 and 475 ms, i.e. between 160 and 130 bpm. Indeed, the individual slope PR/RR (250 data points per heart) was significantly higher in the control group (0.53 ± 0.27, $n = 5$) than in the ivabradine treated group (0.10 ± 0.04, $n = 5$) ($p = 0.032$). However, during anoxia and reoxygenation no significant correlation could be properly established between PR and RR intervals, most likely due to major electromechanical disturbances, including numerous AV blocks (see Table 1).

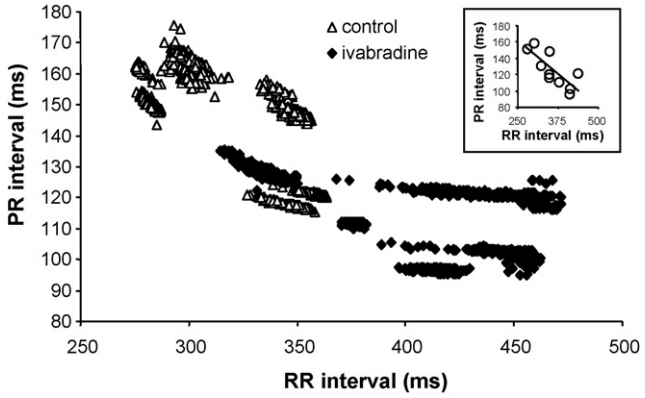

Fig. 5. Beat-to-beat analysis performed in individual hearts showing that the relationship between PR interval and RR interval under normoxic conditions is different in control and ivabradine treated hearts, the PR interval being stabilized by ivabradine. The general linear relationship between PR interval and RR interval is shown in the inset regardless if ivabradine was applied or not ($r^2 = 0.62$). Each beat-to-beat analysis was performed on 250 successive preanoxic cardiac cycles; Ivabradine, 0.3 µM; r^2: coefficient of determination of the linear regression; $n = 5$ hearts in each group.

4. Discussion

4.1. Dose–response curve for ivabradine

The inhibition of the *If* current by ivabradine is known to be specific, selective and use-dependent at concentrations lower than 10 µM. However, at higher concentrations unspecific currents, such as calcium currents (L and T type) that play an important functional role in the embryonic heart, are also inhibited [3]. For this reason, dose–response curves have been determined within the range of 0.1–10 µM of ivabradine. Interestingly, ivabradine lowered embryonic atrial rate in the very same range of concentrations (1–3 µM) and to the same extent (~20%) than in adult sinoatrial node cells [4,20], corresponding to half-block concentrations for HCN1 and HCN4 subtypes [20,21]. These observations indicate that the pharmacological modulation of HCN channels could be similar in embryonic and adult pacemaker cells, which deserves to be further investigated. Although ivabradine (1 µM) decreased the beating rate by about 40 bpm both in atria and ventricle (equal effect), it was more efficient in ventricle than in atria, i.e. a drop of frequency of 50% versus 20%. Furthermore, compared with other regions, ivabradine had a stronger inhibitory effect on spontaneous activity of the outflow tract, even at the lowest concentration.

Concentrations of ivabradine higher than 0.3 µM decreased further atrial rate but had no additional effect on ventricular rhythm, suggesting that non-HCN pacing mechanisms, such as sodium–calcium exchanger [22] and intracellular calcium release [23,24], can also contribute to pacemaker current more markedly in the ventricle than in atria of the embryonic heart. Our experiments showing that spontaneous contractions of the isolated outflow tract were totally abolished by ivabradine, in contrast to atria and ventricle, support the hypothesis that pacemaking activity in this part of the heart rely on HCN channels exclusively, and including at least HCN4.

Under normoxia, ivabradine had a negative chronotropic effect without any inotropic effect, alike in adult sinoatrial myocytes [4]. Thus, the fact that inhibition of HCN channels slowed the

spontaneous beating rate differently in the three cardiac regions and that the HCN4 isoform was inhomogenously distributed, suggest a subtle gradient of density and/or properties of HCN channels along the developing heart.

4.2. Anoxia-reoxygenation

The embryonic heart at the investigated stage is not innervated by the autonomic nervous system and no neurohumoral influence exists under our in vitro conditions. Thus, the observed changes of pacemaker rate during anoxia-reoxygenation were exclusively regulated at the cardiomyocytes level.

In addition to its negative chronotropic effect, inhibition of HCN channels appears to stabilize atrio-ventricular conduction, especially when heart rate is below 160 bpm, under normoxia (Fig. 5) as well as during anoxia and reoxygenation (Fig. 4). To our knowledge, such a dromotropic effect of HCN channel inhibition has not been reported in adult heart [5,25]. It should be noticed that this PR interval stabilizing effect can be clearly observed with ivabradine, but it may not be exclusively due to HCN inhibition.

It has been shown [26] that if HCN channels are blocked, cells tend to hyperpolarize more which in turn relieves inactivation of more Ca^{2+} and/or Na^{+} channels involved in action potential propagation and consequently improves conduction. This hypothesis is strongly supported by the evident correlation observed between PR interval and RR interval. A complementary interpretation is that Ca^{2+} reuptake into sarcoplasmic reticulum and transsarcolemmal Ca^{2+} extrusion, taking place during a significantly prolonged diastole (+25%), could somewhat lower basal diastolic intracellular calcium relative to untreated hearts. This is consistent with a slight enhancement of gap junction permeability by a lower intracellular Ca^{2+}. Indeed, although there is no specialized conduction system at the stage investigated, various connexins (e.g. Cx43) forming gap junctions are expressed and functional during early cardiogenesis [27,28]. Furthermore, alike in adult heart, decreasing heart rate reduces myocardial oxygen consumption in the embryonic chick heart at stage 24HH [29], which is known to protect heart function especially during an ischemic/hypoxic episode. Remarkably, during anoxia and reoxygenation, inhibition of HCN channels did not affect ventricular depolarization–repolarization (QT duration), excitation–contraction coupling (EMD) and contractility (shortening) and had no additional proarrhythmic effects as those observed in the adult mouse heart model at highly unspecific ivabradine concentrations [30]. During the last 20 min of anoxia QTc was 23% longer in ivabradine treated hearts than in control hearts but no additional arrhythmias were observed. Although a longer QTc puts an adult heart at increased risk for arrhythmias, it does not seem to be the case in the minute embryonic heart at stage 24HH in which the compact myocardium of the ventricle is very thin (200–300 μm) and action potential duration quite uniform in space.

Although a tendency toward a shorter time to resumption in atria and ventricle of treated hearts relative to control hearts can be observed in Table 1, there was no significant difference between the experimental groups. It should be noticed that the relatively important interindividual variability of the investigated parameters is probably due to slight differences in developmental stage, level of differentiation, cardiac dimensions and degree of flattening of the hearts in the culture compartment as previously discussed [17]. Moreover, the unavoidable arrhythmic activity induced by anoxia and reoxygenation was also responsible for a great variability. For example, in Fig. 4, the fact that atrial rate during anoxia was the average rate determined during bursts of activity contributed to increase interindividual variability. Nevertheless, the preanoxic PR and QT values were within the range found in our previous work [16].

4.3. Distribution of the HCN4 channel

The antibody against HCN4 identified a band at about 160 kDa, corresponding to molecular weight of HCN4 which predominates over the other HCN isoforms in the adult sinoatrial node [31]. Our data provide the first direct evidence that the HCN4 channel, considered as the predominant isoform during early cardiogenesis [8–10], is inhomogeneously distributed within the heart of the chick embryo, i.e. atria = ventricle > outflow tract. Ivabradine at 10 μM reduced the beating rate in both atria and ventricle by about 40 bpm but abolished spontaneous activity of the outflow tract. These findings support the concept that the low intrinsic beating rate of the outflow tract can be attributed to the low density of HCN4 channels. As the fate of the outflow tract is to differentiate at older stages into aorta and pulmonary artery rather than spontaneously contracting myocardium, it is not surprising that this otherwise slowly conducting region exhibited the lowest HCN4 expression.

The possibility that other subtypes of HCN channels (i.e. HCN1, 2) blocked by ivabradine and known to be present in the adult heart [2], are expressed in the embryonic heart and play a role in atrial and ventricular pacemaking cannot be ruled out. Furthermore, the spatial distribution of HCN4 drastically changes throughout cardiac development [8,32] and HCN4 can heteromerize with HCN2 to form functional pacemaker channels in the embryonic heart [33].

5. Conclusion

The gradient of intrinsic beating rate within the embryonic heart model, the differential efficiency of ivabradine in the sinoatrial region, ventricle and outflow tract and the characteristic distribution of the predominant HCN4 isoform along the heart, indicate that HCN channels play an important role in the fine control of the rate of diastolic depolarization in all cardiac regions. Moreover, our findings show that HCN channels can be involved in the chrono- and dromotropic responses to transient oxygen deprivation without affecting E–C coupling or contractility.

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