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Age-dependent changes in ion channel mRNA expression in canine cardiac tissues

Mónika Gönczi[1], Péter Birinyi[1], Bernadett Balázs[1], Norbert Szentandrássy[1], Gábor Harmati[1], Zoltán Könczei[2], László Csernoch[1] and Péter P. Nánási[1]

[1] *Department of Physiology, University of Debrecen, Debrecen, Hungary*
[2] *K. Z. Veterinary Ambulance, Domb u. 16, Debrecen, Hungary*

Abstract. The expression pattern of cardiac ion channels displays marked changes during ontogeny. This study was designed to follow the developmental changes in the expression of major ventricular and atrial ion channel proteins (including both pore forming and regulatory subunits) in canine cardiac tissues at the mRNA level using competitive reverse transcription polymerase chain reaction. Therefore, the corresponding mRNA levels were compared in myocardial tissues excised from embryonic (25–60 days of gestation) and adult (2–3 years old) canine hearts. Expression level of Kv4.3, Kv1.4, KChIP2, KvLQT1, and Cav3.2 mRNAs were higher in the adult than in the embryonic hearts, while expression of Nav1.5 and minK mRNAs were higher in the embryonic than in the adult myocardium. No change in Kir2.1, HERG, Kv1.5, and Cav1.2 mRNA was observed during ontogeny. Direction of the developmental change in the mRNA level, determined for any specific channel protein, was identical in the atrial and ventricular samples. The age-dependent increase observed in the expression of Kv4.3, Kv1.4, KChIP2, and KvLQT1 is congruent with the greater repolarization reserve of the adult myocardium, associated with higher densities of I_{to} and I_{Ks}. The results indicate that age-dependent changes in the expression pattern of many ion channels are similar in canine and healthy human myocardium, therefore, canine cardiac muscle can be considered as a good model of studying developmental changes in the human heart.

Key words: Developmental changes — Dog heart — Ion channels — mRNA expression — Regional differences

Introduction

Configuration of the action potential, together with the density and properties of the underlying ion channels, is dramatically changing during the embryonic life. Most of these alterations extend into the early, and sometimes to the later postnatal period. The earliest relevant data were obtained from embryonic chick ventricular cells indicating that the density of some ion currents, like I_{Na} and I_{K1} increased, while the density of others, such as $I_{Ca\text{-}L}$, decreased with ontogeny in the prenatal period (Fujii et al. 1986; Sperelakis 1989; Tohse et al. 1992; Sada et al. 1995). Later these developmental studies were extended to cardiac preparations obtained from various mammalian species, including rats, mice, rabbits, and guinea pigs. The results showed serious interspecies differences, suggesting that extrapolation of the ontogeny results from one species to another may be quite hazardous in the case of many ion currents. Regarding therapeutic relevance, human ontogeny data would be ideally required, however, we have only limited access to human embryonic heart tissues, in addition, these are almost exclusively atrial samples (Cohen and Lederer 1993; Mansourati and Le Grand 1993; Crumb et al. 1995; Roca et al. 1996). Data on embryonic human ventricular samples are restricted to action potential recordings (Jezek et al. 1982, 1984, 1985). This is the reason why we have chosen canine cardiac tissues (both ventricular and atrial ones) in the present developmental study, since canine heart is considered to be the best model of the human heart in terms of its electrophysiological properties, including action potential morphology and the set of the underlying ion currents (Szabó et al. 2005; Szentandrássy et al. 2005). In spite of the widespread interspecies differences found in the

Correspondence to: Péter P. Nánási, Department of Physiology, University of Debrecen, H-4012 Debrecen, P.O.Box 22, Hungary
E-mail: nanasi@phys.dote.hu

literature, our results obtained in developing canine cardiac tissues at the mRNA level are largely congruent with those of the corresponding human voltage clamp measurements, indicating that the density of I_{to} is increasing (Mansourati and Le Grand 1993; Crumb et al. 1995), while that of I_{K1} (Crumb et al. 1995) and $I_{Ca\text{-}L}$ (Cohen and Lederer 1993; Roca et al. 1996) is not changing during the ontogeny of canine and human hearts. The importance of developmental studies on ion channel expression may be verified by the changes of ion current densities observed during electrical remodeling caused by a variety of pathological states. These changes are reciprocal to those seen during the ontogeny, i.e. the embryonic patterns are reintroduced in the diseased hearts.

Materials and Methods

Cardiac muscle samples were isolated from the atrial and ventricular myocardium of dogs with different ages. Canine embryonic heart tissues (25–60 days of gestation) were collected after surgical removal of uterus, while adult samples were obtained from 2–3 years old animals euthanized with intravenous ketamine hydrochlorid (Calypsolvet 10 mg·kg^{-1}) and xylazine hydrochlorid (CP-xylazin 1 mg·kg^{-1}). The entire investigation was carried out in accordance with the EU (86/609/EEC) guidelines, and was approved by the local ethical committee.

After the tissue samples were removed from the appropriate part of the heart, they were fast-frozen in liquid nitrogen and stored at –80°C. Total RNA was isolated from the tissues using RNeasy Plus Mini Kit (Qiagen, Crawley, UK) according to the manufacturer instructions, then RNA concentration of each sample was measured on a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, USA) at a wavelength of 260 nm. Competitive reverse transcription (RT)-polymerase chain reaction (PCR) was used to quantify mRNA expression of the various pore forming and regulatory ion channel proteins. First-strand cDNA was synthesized by RT with canine cardiac RNA samples and oligo$(dT)_{15}$ primer (Promega, Madison, USA) using Omniscript RT Kit (Qiagen, Crawley, UK).

The integrity of each cDNA sample was confirmed by PCR for a general mammalian protein of glucose aldehid phosphate dehydrogenase (GAPDH). 50 µl reaction volume contained the followings (in µl): cDNA: 1, PCR buffer (5x): 10, dNTP Mix (10 mmol·l^{-1}): 1, primer complex (50 µmol·l^{-1}): 1, which included both the forward (5'AATGAGCCCCAGCCTTCTCCAT) as well as the reverse (5'AAGGTCGGAGTCAACGGATTTGG) primer, *Taq* polimerase: 0.25, and nuclease-free water: 36.75. The reaction

Table 1. Parameters of the polymerase chain reactions specific to various ion channels

Ion channel proteins	Annealing temperature (°C)	Product size (bp)	Sequences of the primers	Reference
Kv1.4	53	522	Sense: ATCATTCGTCTGGTCCGAGTATTC Antisense: AACTCCTTCTTCCATCTCTAG	Han et al. 2002
Kv4.3	54	212	Sense: TAGATGAGCAGATGTTTGAGC Antisense: ACTGCCCTGGATGTGGATG	Han et al. 2002
KChip2	55.5 (4×) + 53.5(31×)	239	Sense: GAGGACTTTGTGGCTGG Antisense: CCATCCTTGTTTCTGTCC	Han et al. 2002
Kv1.5	58 (4×) + 56 (31×)	429	Sense: GGCTGACAACCAGGAGACCCA Antisense: GATCGCCGCAAGTCCACATT	*
Kir2.1	56	393	Sense: GACCTGGAGACGGACGAC Antisense: AGCCTGGAGTCTGTCAAAGTC	Yue et al. 1999
HERG	58 (4×) + 56 (31×)	311	Sense: ACGGCGCTCTACTTCACC Antisense: ACCGCGTTCATGTCGATG	Yue et al. 1999
KvLQT1	56	260	Sense: GTCTACAACTTCCTCGAGCGTCCC Antisense: TTCCGGGCAAAGCGCAGC	Han et al. 2002
minK	53.5	179	Sense: CTACATCCGCTCCAAGAAG Antisense: CAGGAAGGTGTGTGTTGG	Han et al. 2002
Nav1.5	54 (5×) + 52 (30×)	314	Sense: TGCATTAACCAGACAGAGG Antisense: CCAATAAAGAGGTTCAGGGTG	Yue et al. 1999
Cav1.2	53	269	Sense: CAATGACACGATCTTCACC Antisense: GGATGCCAAAGGAGATG	Han et al. 2002
Cav3.2	52.5	334	Sense: CTCCTTCCTGCTCATCG Antisense: GTTGCAGTACTTGAGGGC	Han et al. 2002

* primers were designed by PREMIER Biosoft International; bp, basepairs.

mixture was denatured (at 94°C for 3 minutes) and subjected to 35 PCR cycles (denaturation (94°C, 30 s), annealing (56°C, 45 s), and elongation (72°C, 60 s)) followed by a final extension period of 7 min at 72°C. All these PCR reactions were carried out using *Taq* polymerase (Promega). Composition of the reaction volume and the main temperature settings were the same in the gene specific PCR reactions except for the annealing temperature, which was individually determined for each primer pairs (all from Bio-Science, Budapest, Hungary). Parameters of the polymerase chain reactions for each ion channel protein are summarized in Table 1. Absence of genomic contamination of RNA samples was confirmed with reverse transcriptase-negative controls in each experiment.

Electrophoresis of the amplified products was performed on 1.5% agarose gels containing (in mmol/l): TRIS 100, boric acid 100, Na_2EDTA 2, and ethidium bromide. Ethidium bromide fluorescence images were captured using a camera (Kodak Gel Logic 1500 Imaging System, Carestream Health Inc., Rochester, USA) under ultraviolet light. The density of each band was determined using Image-Pro Plus software (Media Cybernetics Inc., Bethesda, USA). Each data from densitometry was normalized to density of the appropriate GAPDH-band. Data were then organized and processed using Microsoft Excel, PowerPoint, and Microcal Origin 6.0 softwares.

Comparisons were made between heart samples obtained from embryonic and adult dogs. From each heart, atrial and ventricular tissues were excised separately and compared to evaluate atrio-ventricular differences in both embryonic and adult hearts. The adult ventricular samples represented the full cross-section of the ventricular wall containing a natural mixture of epicardial, endocardial and (dominantly) midmyocardial tissues. Equal amounts of tissues, dissected from the left and right respective chamber of the heart, were pooled together to exclude distortions due to possible left-right differences. Results are expressed as mean ± SEM values. Statistical significance of differences was evaluated with one-way ANOVA followed by Student's t-test for unpaired data. Differences were considered significant when p was less than 0.05.

Results

Since in the followings, the expression level of each ion channel mRNA will be normalized to that of the housekeeping protein, GAPDH obtained from the same tissue sample, first the age- and tissue-dependent pattern of expression of GAPDH mRNA has to be demonstrated. As indicated in Fig. 1, neither significant atrio-ventricular differences, nor age-dependent changes could be observed. This result is important to verify our analysis based on comparison of ion channel mRNA data normalized to GAPDH mRNA obtained from the same tissue sample.

Transient outward K^+ current (I_{to}) density in the canine heart is determined by two pore forming subunits, Kv4.3

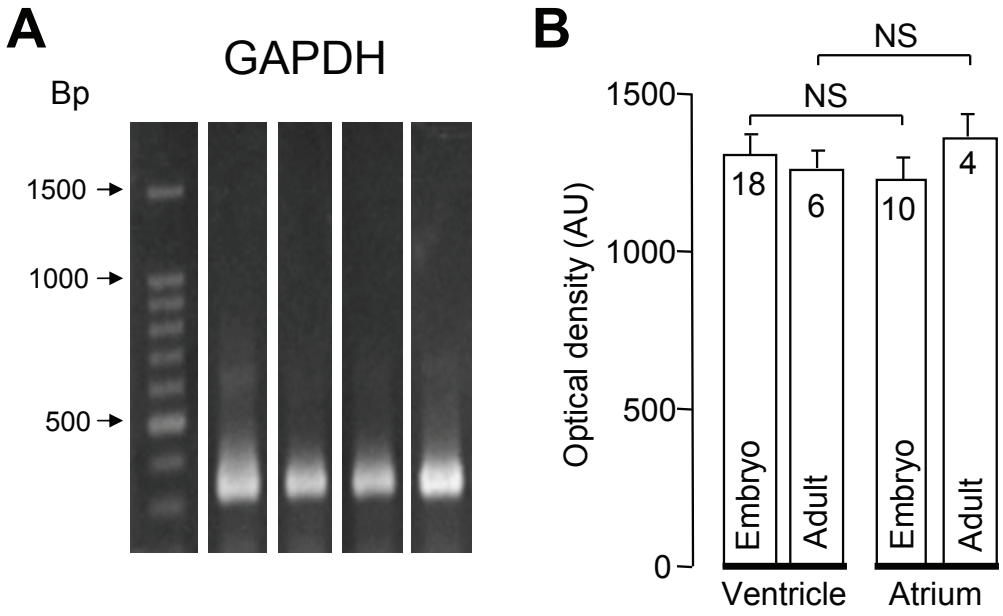

Figure 1. Representative densitogram (**A**) and average data (**B**) indicating the expression level of the housekeeping protein, GAPDH mRNA in embryonal and adult canine atrial and ventricular myocardium. Numbers within the columns indicate the number of samples. Calibration (arrows at left) denotes base pairs (Bp). NS, not significant.

and Kv1.4 (the former being dominant), plus an auxiliary subunit, KChIP2. As demonstrated in Fig. 2A–C, all mRNAs coding these proteins are more abundant in adult than in embryonic myocardium, regardless of the atrial or ventricular origin of the tissues. Comparing the atrial and ventricular samples, Kv4.3 mRNA level was higher in the ventricles than in the atria (in both embryonic and adult tissues), no atrio-ventricular difference was found in the Kv1.4 mRNA level, while KChIP2 density was higher in atrial than ventricular myocardium of the embryonic hearts, in contrast to adult tissues, where this difference was dissipated during the ontogeny.

The delayed rectifier K^+ current family was represented by three independent K^+ currents, namely the rapid delayed rectifier (I_{Kr}), the slow delayed rectifier (I_{Ks}), and the ultra-rapid component (I_{Kur}), with the respective pore forming subunits of HERG, KvLQT1, and Kv1.5. In addition, the auxiliary subunit, minK, is also involved in tuning the density and kinetic properties of I_{Ks}. HERG coding mRNA density showed neither age-dependent changes, nor atrio-ventricular differences (Fig. 3A). Reciprocal age-dependent changes were observed when comparing the density of mRNAs coding for KvLQT1 and minK, as the former increased, while the latter decreased with ontogeny in both atrial and ventricular myocardium, without showing any atrio-ventricular difference (Fig. 3B,C). In contrast, no age-dependent variation was found in the case of Kv1.5 mRNA, but its density was higher in the ventricles than in the atria, independently of its embryonic or adult origin (Fig. 3D). The inward rectifier K^+ current (I_{K1}) is mediated dominantly by Kir2.1 channel protein. Its mRNA level was not altered significantly by the age of the animal, or by the atrio-ventricular origin of the sample (Fig. 3E).

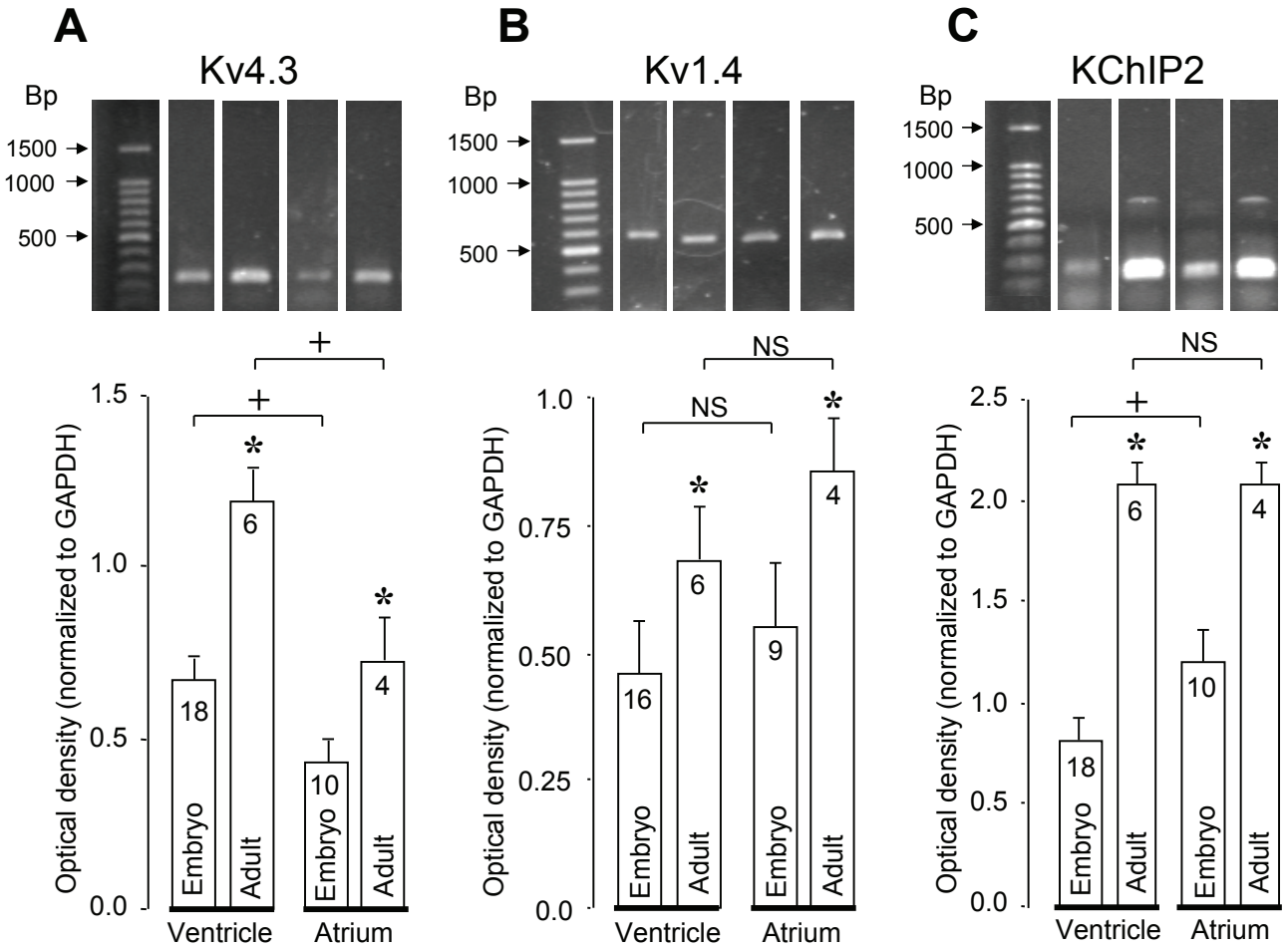

Figure 2. Age-related and atrio-ventricular differences in the mRNA level coding for ion channel proteins related to I_{to} in canine cardiac tissues: Kv4.3 (**A**), Kv1.4 (**B**), KChIP2 (**C**). Representative densitograms are presented in the upper panels, while lower panels show the corresponding average data. * significant age-related differences, $p < 0.05$; + significant atrio-ventricular differences, $p < 0.05$; NS, not significant.

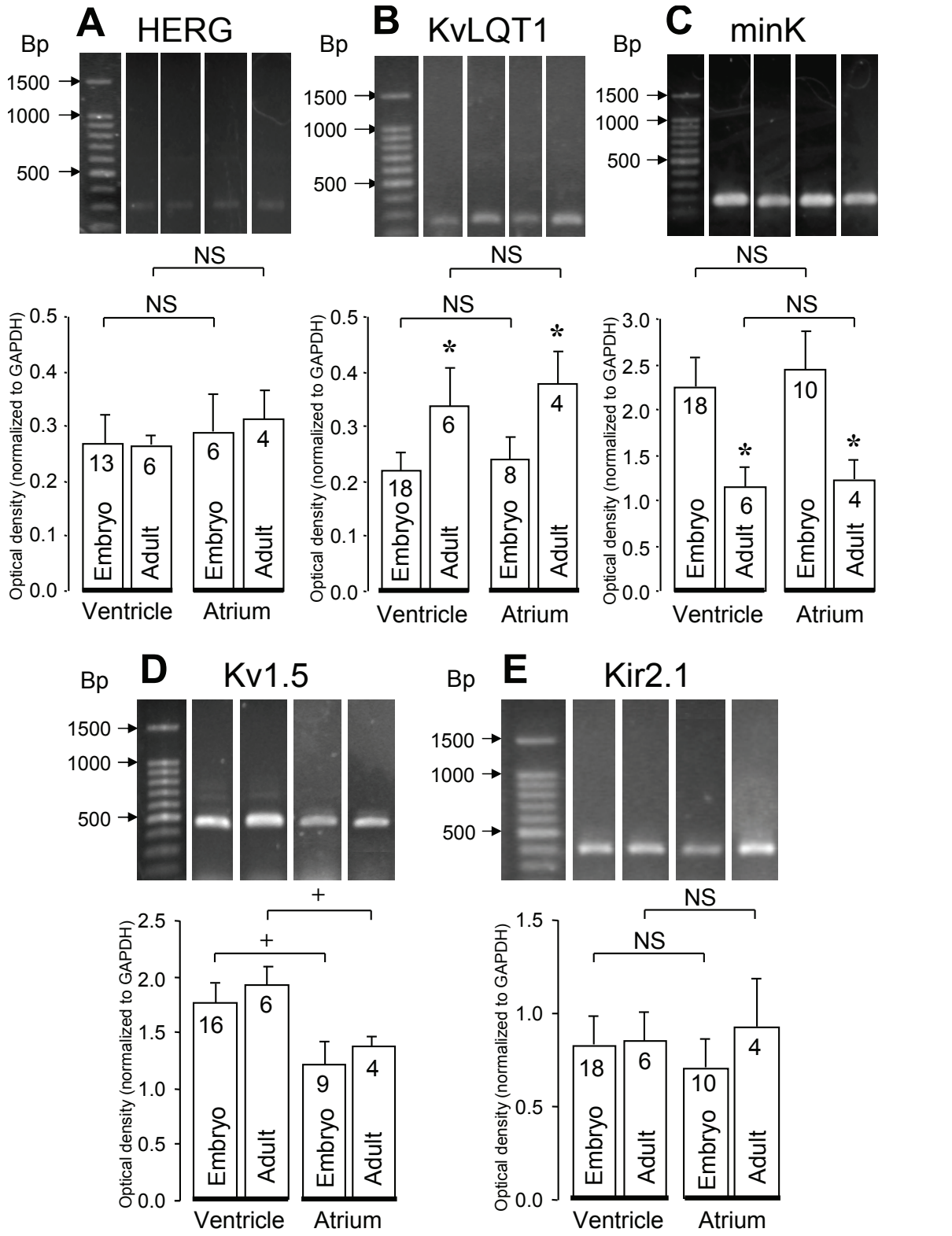

Figure 3. Age-related and atrio-ventricular differences in the mRNA level coding for ion channel proteins related to cardiac delayed rectifier HERG (**A**), KvLQT1 (**B**), minK (**C**), Kv1.5 (**D**) and inward rectifier currents Kir2.1 (**E**). Densitograms and the corresponding average data are presented in the upper and lower panels, respectively. * significant age-related differences, $p < 0.05$; + significant atrio-ventricular differences, $p < 0.05$; NS, not significant.

Age-dependent variability of three pore forming channel subunits, each carrying inward current, was also examined. Fast Na^+ current (I_{Na}), responsible for the upstroke of the action potential, is mediated chiefly by Nav1.5 protein in the canine heart. Nav1.5 mRNA expression level drastically decreased with ontogeny in both atrial and ventricular myocardium (Fig. 4A). Interestingly, embryonic atria contained more Nav1.5 RNA than the age-matched ventricles, while the opposite distribution was observed in the adult hearts (i.e. more RNA was expressed in adult ventricles than in adult atria). Neither age-related, nor atrio-ventricular differences were explored in case of Cav1.2 mRNA, coding for the pore forming subunit of L-type Ca^{2+} channels ($I_{Ca\text{-}L}$, Fig. 4B). On the other hand, significant age-related elevation in the expression level of Cav3.2 mRNA, coding for the pore forming subunit of T-type Ca^{2+} channels ($I_{Ca\text{-}T}$) was observed, without showing atrio-ventricular differences (Fig. 4C).

Discussion

Comparison of present results to human and canine data from the literature

In our experiments, mRNAs coding for ion channel proteins related to I_{to} (i.e. Kv4.3, Kv1.4, and KChIP2) were uniformly higher in adult than in the embryonic canine samples. These results agree well with those of others studying developmental changes in the density of canine I_{to} (Jeck and Boyden 1992; Pacioretti and Gilmour 1995) or the underlying channel proteins (Plotnikov et al. 2004). They are also in line with

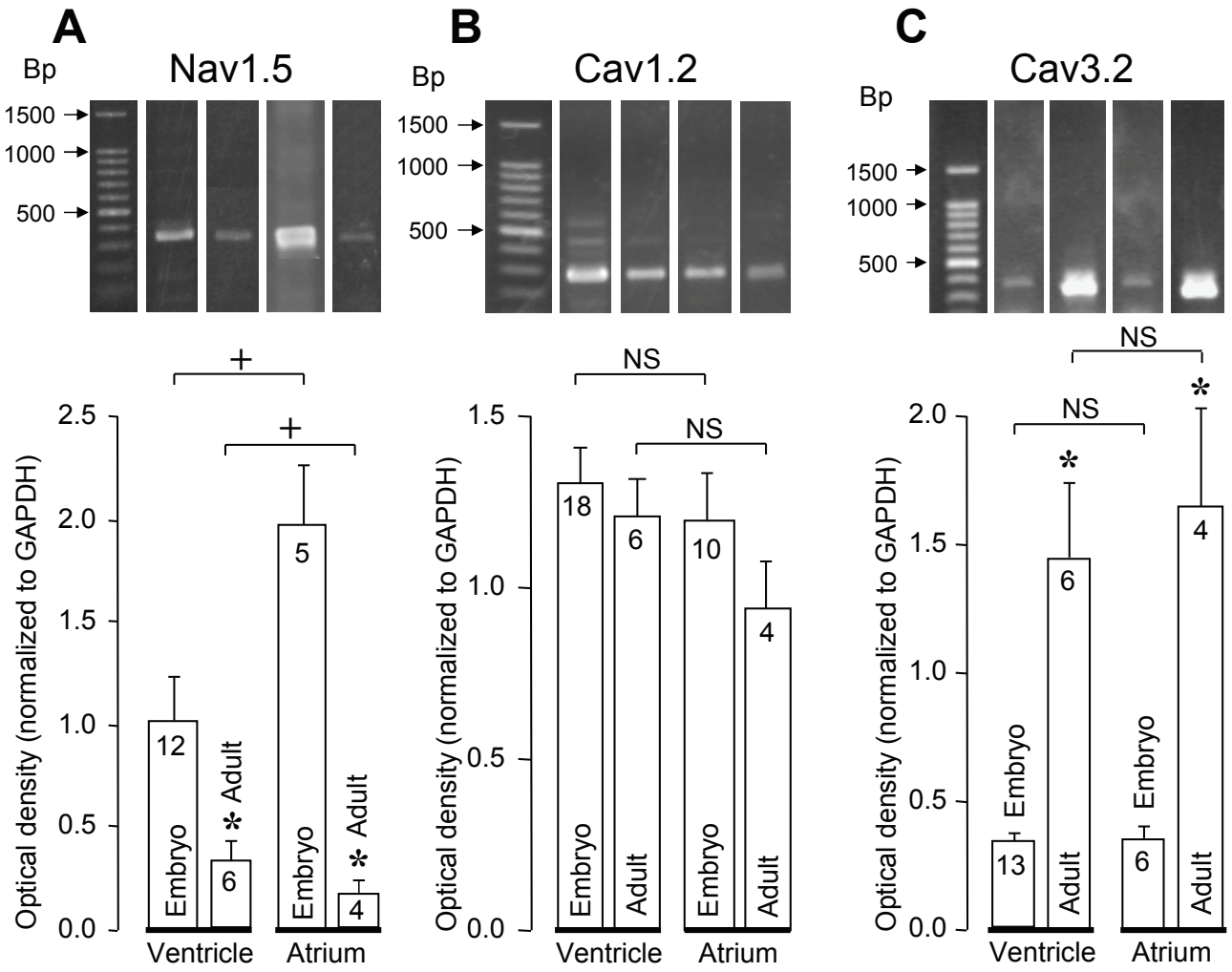

Figure 4. Age-related and atrio-ventricular differences in the mRNA level coding for ion channel proteins mediating inward currents Nav1.5 (**A**), Cav1.2 (**B**), Cav3.2 (**C**). Densitograms with the corresponding average data are shown in the upper and lower panels, respectively. * significant age-related differences, $p < 0.05$; + significant atrio-ventricular differences, $p < 0.05$; NS, not significant.

relevant human data on I_{to}, showing marked increase of the current during ontogeny (Mansourati and Le Grand 1993; Crumb et al. 1995). Similar results were obtained with I_{to} in aging human stem cells (Sartiani et al. 2007).

Regarding the rapid and slow components of the delayed rectifier K^+ current, the density of I_{Kr} decreased, while I_{Ks} increased during the postnatal period of dogs (Obreztchikova et al. 2003; Krishnamurthy et al. 2004). These changes in current densities were accompanied with reduction of HERG, increase in minK, and no change in KvLQT1 expression at both mRNA and protein levels (Krishnamurthy et al. 2004). Although these alterations are not identical with the present results, since we observed an increase in KvLQT1, reduction of minK, and no change in HERG expression, however, they are not exclusive at all. In the above mentioned studies only postnatal alterations were monitored (neonates *versus* adults), while the possible prenatal changes were neglected. This may explain the differences seen between our results and those of Obreztchikova et al. (2003) and Krishnamurthy et al. (2004). No relevant human data on I_{Kr} or I_{Ks} is available for comparison.

We observed no developmental changes in the expression levels of Kir2.1 mRNA, mediating I_{K1}, Cav1.2 mRNA, responsible for $I_{Ca\text{-}L}$, and in Kv1.5 mRNA coding for the ultra-rapid component of I_K, denoted as I_{Kur}. In a good accordance with our results, no change in the density of I_{K1} (Crumb et al. 1995) and $I_{Ca\text{-}L}$ (Cohen and Lederer 1993; Roca et al. 1996) were observed during the ontogeny of human myocardium. Although in a recent human study lower density of baseline $I_{Ca\text{-}L}$ was measured in the atrial myocardium of infants than that of adults, this difference disappeared in the presence of isoproterenol resulting full activation of the current (Tipparaju et al. 2004). Regarding I_{Kur}, this current is abundantly expressed in 8–10 weeks fetal human hearts, however, no comparison to adults is available (Bielanska et al. 2010).

Comparison of present results to those obtained in other mammalian species

Apart from I_{to} and the related channel proteins, which are clearly increasing throughout the various stages of ontogeny in all mammalian species, including dogs (Jeck and Boyden 1992; Pacioretti and Gilmour 1995; Plotnikov et al. 2004), humans (Mansourati and Le Grand 1993; Crumb et al. 1995), rabbits (Sanchez-Chapula et al. 1994; Elizalde et al. 1999), rats (Guo et al. 1997a,b), and mice (Wang and Duff 1997; Harrell et al. 2007), there are unusually extensive interspecies differences regarding the other channel/current systems. For instance, I_{K1}/Kir2.1 was not found to be age-dependent in dogs (present study), humans (Crumb et al. 1995), and guinea pigs (Kato et al. 1996), while its amplitude was shown to increase with age in rabbits (Chen et al. 1991; Huynh et al. 1992), rats (Wahler 1992; Nagashima et al. 2001), and mice (Davies et al. 1996; Grandy et al. 2007).

The pattern of age-related changes is not less conflicting in the case of the three delayed rectifier K^+ currents: I_{Kr}, I_{Ks}, and I_{Kur}. The density of I_{Kr} increased with ontogeny in guinea pigs (Kato et al. 1996), but decreased in mice (Wang et al. 1996, 2000). Expression of HERG mRNA was comparable in embryonic and adult canine hearts (present study), although it was shown to decrease during the postnatal period (Krishnamurthy et al. 2004). I_{Ks} density increased during the ontogeny in guinea pigs (Kato et al. 1996), dogs (Obreztchikova et al. 2003), and mice (Wang et al. 1996; Grandy et al. 2007). The increment of I_{Ks} density, however, this could either be associated with an age-dependent reduction (Harrell et al. 2007; present study) or elevation (Krishnamurthy et al. 2004) of the expression level of minK, and also with an unchanged (Krishnamurthy et al. 2004; Trépanier-Boulay et al. 2004) or increased (present study) density of KvLQT1.

Expression of Kv1.5, responsible for I_{Kur}, increased during the ontogeny in mice (Harrell et al. 2007), decreased in rats (Guo et al. 1997b), while was not age-dependent in dogs (present study). Accordingly, the density of I_{Kur} increased in mice (Grandy et al. 2007) and decreased in rats (Guo et al. 1997a,b) with age.

Regarding inward currents, expression of Cav1.2, the pore forming subunit of $I_{Ca\text{-}L}$, together with the corresponding current density, increased with ontogeny in rabbits (Wetzel et al. 1991, 1993; Osaka and Joyner 1991; Huang et al. 2006; Namiki et al. 2007), guinea pigs (Kato et al. 1996), and mice (Davies et al. 1996; Liu et al. 2002; Harrell et al. 2007; Nguemo et al. 2009), decreased in rats (Cohen and Lederer 1988), while was not age-dependent in humans (Cohen and Lederer 1993; Roca et al. 1996), and dogs (present study). $I_{Ca\text{-}T}$ density and Cav3.2 expression was shown to increase with age in rabbits (Wetzel et al. 1991) and dogs (present study), decrease in mice (Yasui et al. 2005; Harrell et al. 2007), while displayed a biphasic change by first increasing then decreasing in rat atrial myocytes (Xu and Best 1992). I_{Na} density increased with ontogeny in mice (Davies et al. 1996; Harrell et al. 2007). In dogs, Nav1.5 mRNA expression decreased with aging (present study), while expression of Nav1.5 at both mRNA and protein levels displayed a biphasic change by first decreasing then increasing in the sheep (Fahmi et al. 2004).

Atrio-ventricular differences

Direction of the developmental change in the mRNA level, determined for any specific channel protein, was always identical in the atrial and ventricular samples. Comparison of atrial and ventricular mRNAs in the adult samples revealed that for Kv4.3, Kv1.5, and Nav1.5, the expression was lower in the atrial than ventricular tissues. No atrio-ventricular

differences in the mRNA content were found in the case of Kv1.4, KChIP2, HERG, KvLQT1, minK, Kir2.1, Cav1.2, and Cav3.2. Regarding embryonic tissues, atrial expression was higher than ventricular for KChIP2 and Nav1.5. Some of these atrio-ventricular differences (in the expression of Kv1.4, KChIP2, HERG, KvLQT1, minK, Cav1.2, and Nav1.5) are similar in adult canine and human cardiac muscle preparations, while marked differences (in Kv4.3, Kv1.5, Kir2.1, and Cav3.2) were also found (Ördög et al. 2006; Gaborit et al. 2007). Interestingly, all atrio-ventricular differences found in ion current densities in mice (i.e. stronger I_{to} and I_{Kur} in ventricles, while no difference in $I_{K1;}$ Grandy et al. 2007) were identical to those observed in the distribution of the corresponding mRNA expression in the canine preparations of the present study. The most unexpected and striking difference was found in the case of I_{Kur}/Kv1.5, since it was abundantly localized in the atria of human myocardium, while dominantly in the ventricles of dogs and mice.

In summary, our canine mRNA results are largely congruent with human voltage clamp data, indicating that the density of I_{to} and I_{Ks} is increasing, while that of I_{K1} and $I_{Ca\text{-}L}$ is not changing during ontogeny in these species. The age-dependent increase observed in the expression of Kv4.3, Kv1.4, KChIP2, and KvLQT1 is congruent with the greater repolarization reserve of the adult myocardium, associated with higher densities of I_{to} and I_{Ks}, respectively. Based on the present results one may anticipate, that age-dependent changes in the expression pattern of ion channels are similar in canine and healthy human myocardium. Taking into account the extremely wide interspecies variability among the other mammalian species in this regard, the canine cardiac muscle samples can be considered as good models for studying developmental changes in the human heart.

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