Abnormal cardiac repolarization and impulse initiation in German shepherd dogs with inherited ventricular arrhythmias and sudden death

Eugene A. Sosunov, Evgeny P. Anyukhovsky, Alexei Shvilkin, Motoki Hara, Susan F. Steinberg, Peter Danilo Jr., Michael R. Rosen, N. Sydney Moise, Jocelyn Merot, Vincent Probst, Flavien Charpentier, Yves Legeay, Hervé Le Marec

Departments of Pharmacology, Pediatrics and Medicine, College of Physicians and Surgeons of Columbia University, 630 West 168 Street, PH 7 West-321 New York, NY 10032, USA

Department of Clinical Sciences, College of Veterinary Medicine, Cornell University, Ithaca, New York, USA

Laboratoire de Physiopathologie & Pharmacologie Cellulaires & Moléculaires, Centre Hospitalo-Universitaire de Nantes and Laboratoire de Médecine, Ecole, Vétérinaire de Nantes, Nantes, France

Received 2 July 1998; accepted 9 November 1998

Abstract

Objective: We tested the hypothesis that delayed afterdepolarization (DAD)-associated rhythms in German shepherd dogs with reduced anteroseptal left ventricular (LV) sympathetic innervation derive from abnormal β-adrenergic receptor effector coupling. Methods and Results: In anteroseptal LV midmyocardium of afflicted dogs, β-receptor density was greater than that in normal dogs (P<.05), with affinity being equal in both groups. Basal and maximum isoproterenol (ISO) stimulated adenylyl cyclase activity of anteroseptal LV of afflicted dogs was greater than that in normal dogs (P<.05). Isolated anteroseptal M cell preparations of afflicted dogs studied with microelectrodes showed abnormal lengthening, rather than shortening of action potential duration in response to ISO, as well as a 61% incidence of 10^-7 mol/l ISO-induced triggered activity as compared to 12% in normals (P<.05). In contrast, there was no difference between afflicted and control dogs in triggered activity, β-receptors or adenylyl cyclase activity in a normally innervated region of the ventricles. Conclusion: In this model there is an increase in β-receptor density and β-adrenergic stimulation of adenylyl cyclase and of triggered activity in anteroseptal myocardium but not in a normally innervated region of the heart. Hence, abnormal β-adrenergic signal transduction appears associated with the neural abnormality identified in dogs with inherited VT.

Keywords: Autonomic nervous system; Receptors; Ventricular arrhythmias

1. Introduction

Naturally occurring animal models of sudden death (e.g. [1,2]) provide opportunities to identify mechanisms for genetic predisposition to lethal ventricular arrhythmias. Recently, a unique colony of German shepherd dogs with inherited ventricular arrhythmias and sudden death commencing at 4–5 months of age has been described [3]. The arrhythmias range from infrequent unifocal ventricular premature depolarizations through single runs of monomorphic ventricular tachycardia and from multifocal ventricular premature depolarizations through polymorphic ventricular tachycardia and ventricular fibrillation. The dogs show no evidence of structural heart disease. Electrocardiograms, echocardiograms, and serum electrolyte levels do not differ from those of normal dogs; neither do endocardial monophasic action potential durations differ between these and German shepherd dogs having no ventricular arrhythmias [3]. However, there is evidence of inadequate sympathetic innervation in portions of the apical, anterior, septal, and lateral regions of the left

Contributed equally as first author.

E-mail address: emf3@columbia.edu (M.R. Rosen)

Corresponding author. Tel.: +1-212-305-8754; fax: +1-212-305-8351.
ventricle, whereas sympathetic innervation of the posterior and basal regions is normal [4]. These results have led to the suggestion that ventricular arrhythmias in the afflicted dogs may derive, at least in part, from the effects of asymmetrical sympathetic innervation.

The present study was designed to investigate associations between cardiac β-adrenergic receptors and the mechanisms responsible for initiation of ventricular arrhythmias in the afflicted German shepherds. We identified two types of ventricular tachycardia in the intact animal. One, as previously demonstrated [5,6] is pause-dependent and attributable to early afterdepolarizations (EAD); the other is tachycardia-dependent and attributable to delayed afterdepolarizations (DAD) in the setting of an abnormal β-adrenergic signal transduction pathway. Our observations in this model provide insights into the mechanisms that may underlie catecholamine-sensitive and exercise-induced ventricular tachycardias.

2. Methods

2.1. Intact animal studies

Twenty-nine German shepherd dogs were included in the study. The arrhythmia afflicted dogs were from colonies initially bred at Cornell University [3] and maintained at Cornell or in the Veterinary School of Nantes. All studies done at Cornell, Nantes and Columbia were in accordance with institutional guidelines for protection of animals. Electrocardiographic measurements were made on conscious, laboratory-acclimated dogs resting quietly in a sling. Three-lead ECGs (I, II and III) were continuously displayed. The electrophysiologic measurements included heart rate, PR and QT intervals (measured by the method described by Weissenburger et al. [7]). Baseline measurements were made at least 30 min after onset of recordings. Holter monitoring was used to identify spontaneous occurrence of arrhythmias and phenylephrine testing [5] (see below) to identify those animals in which ventricular arrhythmias were inducible by α-adrenergic stimulation.

2.2. Phenylephrine infusion

Nine unafflicted dogs aged 21 to 32 weeks; (30±1 weeks) and six afflicted dogs aged 21 to 31 weeks (28±2 weeks) were used to evaluate the electrophysiologic effects of phenylephrine. Atenolol was injected as an i.v. bolus of 2 mg/kg followed by continuous infusion of 0.3 mg/kg/h. Phenylephrine was injected as four i.v. infusions of increasing doses: 0.1, 0.3, 1, 3, 10 and 30 μg/kg/h (5 min each, see Fig. 4 for protocol). Electrocardiographic measurements were done 5 min after the start of each dose and 5 and 15 min after the end of the last dose.

2.3. Isoproterenol infusion

Eleven unafflicted dogs aged 22 to 100 weeks; (37±10 weeks) and seven afflicted dogs aged 15 to 80 weeks (38±12 weeks) were used to evaluate the electrophysiologic effects of isoproterenol, administered as i.v. infusions of increasing doses: 0.1, 0.3, 1, 3, 10 and 30 μg/kg/h (5 min each, see Fig. 4 for protocol). Electrocardiographic measurements were done 5 min after the start of each dose and 5 and 15 min after the end of the last dose.

2.4. Cellular electrophysiologic studies

Here, we studied not only afflicted and unafflicted German shepherd dogs, but — as additional controls — age- and size-matched mongrels, as well as 18±20-day-old mongrels. We included these mongrel groups for two reasons: first, to permit a comparison of control German shepherds with a different population of normal dogs, and second, to determine if there were developmental changes in electrophysiologic properties that would help us understand the abnormalities in the afflicted German shepherds. This was of particular importance as our earlier work (see Section 4) tested the association of the evolution of cardiac sympathetic innervation with changes in electrophysiologic actions of adrenergic agonists.

Animals were anesthetized with sodium pentobarbital, 30 mg/kg i.v. (4±5 months old) or 40 mg/kg i.p. (18±20 days old). The hearts were removed quickly through a left lateral thoracotomy and immersed in cold Tyrode’s solution equilibrated with O₂–CO₂ (95:5) and containing (in mmol/l): NaCl 131, NaHCO₃ 18, KCl 4, CaCl₂ 2.7, MgCl₂ 0.5, NaH₂PO₄ 1.8, and dextrose 5.5. Transmural slabs (~0.1-cm thick) were filleted with surgical blades perpendicular to the surface of the anteroseptal and postero basal left ventricular wall [8–10] to permit impalement of midmyocardial (M) cells, which were used as a representative ventricular myocardial cell type. Purkinje fibers were obtained from left and right ventricles of three afflicted and eight unafflicted German shepherd dogs, respectively, 56 to 80-weeks-old (63±4 weeks) and 37 to 61-weeks-old (49±3 weeks, P>.05 vs. unafflicted).

All preparations were placed in a tissue bath, superfused with Tyrode’s solution warmed to 37±0.5°C (pH 7.3±7.4), and allowed to equilibrate at a cycle length (CL) of 1000 ms. Solutions were pumped through the bath at 12 ml/min, such that chamber content changed three times a minute. The bath was connected to ground via a 3 M KCl/Ag/
AgCl junction. Transmembrane potentials were recorded using 3 M KCl-filled glass capillary microelectrodes (tip resistances of 10–20 MΩ) coupled by an Ag/AgCl junction to an amplifier with a high-input impedance and input capacity neutralization (model KS-700, World Precision Instruments). \( V_{\text{max}} \) as obtained by electronic differentiation was displayed on a digital storage oscilloscope (model 4074, Gould) and stored in digitized form in a personal computer for subsequent analysis. For stimulation of preparations, standard techniques were used to deliver 0.5–2 ms long square-wave pulses 2.0 times threshold through bipolar PTFE-coated silver electrodes [11].

Experiments were started after preparations displayed stable electrophysiological characteristics, requiring 60 min for Purkinje fibers and 3–4 h for transmural slabs. Impalements of Purkinje fibers and of myocardium were made at comparable sites in afflicted and normal animals to minimize the possibility that differences in anatomical location would confound the results. We measured the maximal diastolic potential (MDP), the maximum upstroke velocity of phase 0 of the action potential (\( V_{\text{max}} \)), the action potential amplitude and the action potential duration at 30% (\( APD_{30} \)), 50% (\( APD_{50} \)) and 90% (\( APD_{90} \)) of full repolarization. Before pharmacological interventions, control steady-state dependence of action potential parameters on the CL of stimulation was determined. The CLs used were 4000–300 ms for the transmural slabs and 8000–300 ms for the other tissues. Each frequency was maintained for 5 min before collecting data. The CL was then returned to 1000 ms until the next frequency scan was performed. Graded concentrations of phenylephrine (10\(^{-8}\)–10\(^{-6}\) mol/l) and isoproterenol (10\(^{-9}\)–10\(^{-7}\) mol/l) were studied in some preparations with 60 min washout permitted between the two agonists. The preparations were allowed to equilibrate for 10 min at each agonist concentration before the frequency scan was performed. To test for the presence of DADs, tissues were stimulated for 1 min at a CL of 250 ms and then stimulation was discontinued.

2.5. Biochemical studies

2.5.1. Membrane preparation

Tissues were trimmed of fat and connective tissue, weighed, minced, and homogenized twice for 10 s in 4 volumes (w/v) of ice-cold homogenization buffer (0.25 mol/l sucrose, 0.03 mol/l histidine, and 1 mmol/l EDTA) with a Polytron (Brinkman). The crude homogenate was centrifuged at 1500 g for 15 min to remove large tissue fragments, nuclear debris, and cellular organelles. The supernatant was centrifuged at 43 000 g for 45 min, and the pellet resuspended in homogenization buffer at a protein concentration of 3–5 mg/ml. Membranes were stored in aliquots at –70°C. The average membrane protein yield was ~0.15–0.30% of the initial tissue wet weight. Membrane fractions prepared in this manner retain stable receptor binding and adenylyl cyclase activity for at least 2 months but were typically used within 3 weeks of preparation.

2.5.2. β-Adrenergic receptor binding assay

\([\text{\textsuperscript{125}}\text{I}]\text{Iodocyanopindolol (ICYP theoretical specific activity of 2200 Ci/mmol) was purchased from NEN. Binding assays were performed essentially as described previously [12]. Briefly, myocardial membranes (35 μg) were incubated for 60 min at 37°C with ICYP (4 to 250 pmol/l in a final volume of 1 ml) The assay buffer contained 0.15 mol/l NaCl, 0.01 mol/l KCl, 0.01 mol/l MgCl\(_2\), 0.001 mol/l EDTA, 2 mg/ml dextrose, 1 mg/ml bovine serum albumin, and 0.01 mol/l Tris, pH 7.4. Preliminary studies verified that the reaction was linear with protein and was at equilibrium under these assay conditions. ICYP bound to membrane protein was separated from free, unbound ICYP by rapid vacuum filtration of the entire 1-ml assay volume over glass-fiber filters (Gelman) followed by one wash with 10 ml of 10 mmol/l Tris, pH 7.4. Radioactivity trapped by the filters was detected with a Packard Autogamma scintillation spectrophotometer. Specific binding of ICYP, defined as the component of total binding inhibited by excess unlabeled propranolol (1 μmol/l), constituted ≈85–90% of total binding at concentrations of ICYP near the equilibrium dissociation constant (\( K_d \)). The maximal number of binding sites (\( B_{\text{max}} \)) and the \( K_d \) value for ICYP were determined by Scatchard analysis of saturation binding isotherms.

2.5.3. Adenylyl cyclase assay

Adenylyl cyclase activity was determined in an assay that monitors the conversion of \([\alpha-\text{\textsuperscript{32}}\text{P}]\text{ATP to cyclic [\text{\textsuperscript{32}}\text{P}]AMP as described previously [12] Incubation mixtures contained Tris (0.05 mol/l, pH 7.5), ATP (0.143 mmol/l), an ATP-regenerating system (10 μg creatine phosphate and 14 μg creatine phosphokinase), theophylline (8 mmol/l), MgCl\(_2\) (2.5 mmol/l), KCl (10 mmol/l), \([\alpha-\text{\textsuperscript{32}}\text{P}]\text{ATP (1–2–10^6 cpm per assay tube), and membrane protein (4 μg). MnCl\(_2\), forskolin, Gpp(NH)p, and isoproterenol were added at the concentrations needed in individual experiments. Assays were performed in triplicate for 30 min at 37°C in a final volume of 75 μl. Reactions were terminated by the addition of 100 μl of cold stopping solution containing 4.5 mmol/l ATP, 1.4 mmol/l unlabeled cAMP, and 50 000 cpm cyclic [\text{\textsuperscript{3}}\text{H}]AMP as an internal standard. cAMP was isolated by sequential Dowex and alumina chromatography. cAMP recovery, as assessed by the recovery of cyclic [\text{\textsuperscript{3}}\text{H}]AMP, typically ranged from 75 to 85%.

2.6. Statistical analysis

Microelectrode data were analyzed from impalements
maintained throughout the course of each experimental protocol. Data are expressed as mean±S.E.M. The statistical method used was one- or two-way ANOVA for multiple groups or for repeated measures, with Bonferroni’s test when the F value permitted this [13]. Significance of incidence of extrasystoles was evaluated with Fisher’s exact test. Significance was determined at P<.05.

3. Results

3.1. ECG recordings

Fifteen of twenty-nine German shepherd dogs used in the study were determined to be afflicted via documentation of spontaneous or phenylephrine-induced ventricular tachycardia. In contrast, no control dogs manifested ventricular tachycardia. Ten afflicted dogs had pause-dependent ventricular tachycardia and five afflicted dogs had sinus tachycardia-induced ventricular tachycardia (see Fig. 1 for example). We used previously described methods [14,15] to describe the relationship between the CL of the last sinus beat preceding an ectopic complex and the coupling interval of the ectopic complex for those arrhythmias that were tachycardia-induced. As noted in the example in Fig. 2, as sinus CLs decreased so did the coupling interval of the first ectopic beat. Moreover, at longer CLs there were fewer or no ectopic beats in these animals. These results are consistent with DAD-induced triggered activity as a cause of the arrhythmia [14,15].

Finally (see Figs. 3 and 4), the only significant electrocardiographic difference between the two groups was a significantly greater PR interval in the afflicted dogs.

3.2. Electrocardiographic effects of phenylephrine

Phenylephrine had similar effects on heart rate in unafflicted and afflicted dogs (Fig. 3A). The highest doses of phenylephrine (1 and 3 mg) significantly decreased heart rate in both groups. Phenylephrine effects on the QT interval were also similar in both groups (Fig. 3C), with the maximum increase being 14 ms in unafflicted dogs, and 8 ms in afflicted dogs.

Fig. 3B shows that phenylephrine prolonged the PR interval more in afflicted than in unafflicted dogs during the 1 and 3 mg/kg/h infusions. A maximum increase of PR interval occurred 5 min after the start of the 3 mg/kg/h infusion. Compared to values recorded after β-blockade (15 min after the i.v. bolus of atenolol), this maximum PR

![Fig. 1. Electrocardiographic recordings from two afflicted German shepherd dogs illustrating pause-dependent (dog s235) and pause-independent (dog s230) spontaneous development of ventricular arrhythmias.](https://academic.oup.com/cardiovascres/article-abstract/42/1/65/325668)
interval increase was four times greater in afflicted than in unafflicted dogs.

Arrhythmias were seen in only one control dog. This was an idioventricular escape rhythm (35 bpm) that occurred at the highest dose of phenylephrine (3 mg/kg/h), and disappeared during washout. In all afflicted dogs, phenylephrine induced pause-dependent ventricular extrasystoles (up to 5/min) or ventricular tachycardia, regardless of whether or not these occurred during control monitoring.

3.3. Electrocardiographic effects of isoproterenol

As shown in Fig. 4A, isoproterenol effects on heart rate were similar in unafflicted dogs and afflicted dogs. We observed an increase in heart rate at the 3, 10 and 30 μg/kg/h doses. Isoproterenol shortened the PR interval more in afflicted (from 113±8 ms to a minimum of 86±6 ms) than in unafflicted dogs (from 101±4 to a minimum of 84±2 ms) (Fig. 4B). Significant shortening was seen at 3, 10 and 30 μg/kg/h. The PR interval was longer in control than in afflicted dogs and attained equivalence to the controls during the 3, 10 and 30 μg/kg/h infusions. The maximum decrease of PR was two times greater in afflicted dogs. Finally, isoproterenol shortened the QT interval in both groups of dogs comparably (see Fig. 4C), the maximum decrease being 36 ms in unafflicted dogs and 39 ms in afflicted dogs.

In five unafflicted dogs, the isoproterenol-induced increase in heart rate was associated with the occurrence of atrial and ventricular extrasystoles (less than 5/min at 10 and 30 μg/kg/h), that disappeared during washout. The arrhythmogenic effects of isoproterenol were more complex in the afflicted dogs. Four dogs had no arrhythmias under control conditions at the time of the experiment. In two of these, isoproterenol increased sinus rate only. In the two others, isoproterenol induced sinus tachycardia associated with ventricular extrasystoles or ventricular tachycardia (>10 min) that disappeared during washout. The other afflicted dogs developed sinus tachycardia associated with ventricular premature depolarizations or bursts of ventricular tachycardia during isoproterenol infusion. For those dogs which also had manifested pause-dependent arrhythmias during control, these arrhythmias disappeared during the sinus tachycardia that accompanied infusion of isoproterenol and recommenced during washout, at which time incidence was greater than or equal to control.

3.4. Studies of isolated cardiac tissues

All afflicted dogs studied in these protocols displayed ventricular premature depolarizations and/or ventricular tachycardia (four or more ventricular complexes in a row) during control ECG recordings. Pause-dependent as well as tachycardia-induced ventricular ectopic activity had been observed in their ECGs.

3.5. Purkinje fibers

These experiments were performed largely as controls,
Fig. 3. Effects of increasing doses of phenylephrine (1, 0.1 mg/kg/h; 2, 0.3 mg/kg/h; 3, 1 mg/kg/h and 4, 3 mg/kg/h) on heart rate (HR) (A), PR interval (B) and QT interval (QT) (C) in unafflicted (n=9, ○) and afflicted German shepherds (n=6, △). The experiment was performed under β-blockade consisting of an i.v. bolus injection of atenolol (arrow) followed by an infusion during the entire experiment. Each dose of phenylephrine was infused for 5 min.

Fig. 4. Effects of increasing doses of isoproterenol (1, 0.1 μg/kg/h; 2, 0.3 μg/kg/h; 3, 1 μg/kg/h; 4, 3 μg/kg/h; 5, 12 μg/kg/h and 6, 30 μg/kg/h) on heart rate (HR) (A), PR interval (B) and QT interval (QT) (C) in unafflicted dogs (n=11, ○) and afflicted dogs (n=7, △). Each dose of isoproterenol was infused for 5 min.

to relate our present results to those of earlier investigators [6]. At CL=1000 ms, Purkinje fibers from afflicted and control dogs, respectively, showed no differences in MDP (−91±1 and −92±1 mV) action potential amplitude (123±3 vs. 129±1 mV) and $V_{\text{max}}$ (500±37 vs. 490±29 V/s). The major differences between the groups occurred in APD. As shown in Fig. 5A, whereas duration did not differ significantly at short CLs, at those >1000 ms, duration was significantly longer in the afflicted animals. Fig. 5B shows representative action potentials from a control (left) and an afflicted (right) dog. Note the greater increase in APD at the longer CL in the afflicted animal. As shown in an earlier study [6], EADs were observed in Purkinje fibers from afflicted dogs. The incidence of EAD increased with prolongation of CL; they were not seen at CL<1000 ms and they were observed in 11, 22, 67 and 80% of preparations at CL of 1000, 2000, 4000 and 8000 ms, respectively, with EAD occurring over the entire age range of study (i.e. 20 through 80 weeks of age).

Isoproterenol had no significant effects on MDP, action potential amplitude and $V_{\text{max}}$ in Purkinje fibers from either
Afflicted or control dogs at 20–25 weeks of age. It induced a concentration-dependent shortening of the APD in both groups of fibers at all CLs from 330–2000 ms that did not differ across groups (data not shown). Fibers could not be stimulated at longer CLs because of isoproterenol-induced automaticity.

In seven Purkinje fibers from afflicted 20–25-week-old dogs that had no EAD at CL 2000 ms, phenylephrine increased the incidence of EAD in a dose-dependent fashion. EAD were observed in 28% of fibers at $10^{-7}$ mol/l and in 57% of fibers at $10^{-6}$ mol/l phenylephrine. No EADs were seen in Purkinje fibers from control dogs at any CL and phenylephrine concentration. Examples of phenylephrine effects are shown in Fig. 6.

Two additional afflicted dogs were studied after the complete disappearance of their arrhythmias, at more than 2 years of age (ages 125 and 240 weeks). In these dogs (seven fibers), at a CL of 1000 ms, APD$_{90}$ was not significantly different from that in unafflicted dogs ($314\pm16$ and $304\pm12$ ms, respectively). Neither increasing the CL nor superfusion with phenylephrine induced the occurrence of EADs. These results suggest that the disappearance of arrhythmias coincides with the normalization of cellular repolarization.

### 3.6. Midmyocardial (M) cells

In these experiments we included, as an additional control group, mongrel dogs age- and size-matched to the German shepherds, and — to identify developmental changes — 18–20-day-old mongrels. We viewed the latter group as important, as we previously have shown the effect of β-agonist on the action potentials of neonatal hearts prior to innervation to be blunted (see below for discussion). Hence, a comparable β-response of normal neonatal and of afflicted hearts would be consistent with a failure of innervation to develop in the latter group.

There were no significant differences in action potential characteristics of M cells between the anteroseptal and
Table 1
Action potential parameters measured at a CL of 1000 ms in transmural myocardial slabs isolated from the anterior septal and posterobasal regions of the left ventricle of normal mongrel and German shepherd dogs, normal mongrel neonates and afflicted German shepherds

<table>
<thead>
<tr>
<th></th>
<th>Afflicted German shepherd</th>
<th>Control mongrel</th>
<th>Control German shepherd</th>
<th>Neonatal mongrel</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Anteroseptal</td>
<td>Posterobasal</td>
<td>Anteroseptal</td>
<td>Posterobasal</td>
</tr>
<tr>
<td>MDP (mV)</td>
<td>89±2</td>
<td>90±2</td>
<td>89±2</td>
<td>90±2</td>
</tr>
<tr>
<td>Amp (mV)</td>
<td>115±2</td>
<td>117±2</td>
<td>114±2</td>
<td>116±2</td>
</tr>
<tr>
<td>( V_{\text{max}} ) (V/s)</td>
<td>282±15</td>
<td>284±22</td>
<td>276±18</td>
<td>302±16</td>
</tr>
<tr>
<td>APD(_{50}) (ms)</td>
<td>220±6</td>
<td>225±6</td>
<td>216±7</td>
<td>223±7</td>
</tr>
<tr>
<td>APD(_{90}) (ms)</td>
<td>266±7</td>
<td>274±7</td>
<td>260±7</td>
<td>267±8</td>
</tr>
</tbody>
</table>

Values are mean±S.E.M. (\( n=12 \) for mongrel and shepherd, \( n=10 \) for puppies). 
Amp, action potential amplitude; MDP, maximum diastolic potential; APD\(_{50}\) and APD\(_{90}\), action potential duration at 50% and 90% repolarization, respectively; \( V_{\text{max}} \), maximum rate of rise of action potential upstroke. 
* \( P<.05 \) vs. the corresponding region of all other groups.

Posterobasal regions of 20–25-week-old animals as well as between afflicted German sheep and control mongrel and German shepherd dogs. Table 1 illustrates this result at a CL of 1000 ms. Whereas amplitude of phase 0 and MDP of M cells from neonates did not differ from those of control 20–24-week-old dogs, APD was shorter and \( V_{\text{max}} \) was lower (both \( P<.05 \)). Phenylephrine (from \( 10^{-8} \) to \( 10^{-6} \) mol/l) had no effects on any action potential parameter at all CLs in all groups of preparations (data not shown).

Fig. 7 illustrates representative M cell transmembrane potentials in all groups of animals recorded at a CL of 4000 ms in control and in the presence of \( 10^{-7} \) mol/l isoproterenol. Isoproterenol had no effects on MDP, action potential amplitude and \( V_{\text{max}} \) in all preparations. APD and plateau level were changed significantly by isoproterenol and these effects depended on region, type of animal and CL. Data summarizing the effects of varying the concentration of isoproterenol in all experiments at three CLs are shown in Fig. 8. At CLs of 300 and 1000 ms, isoproterenol did not alter APD significantly in any preparations. At a CL of 4000 ms, the effects of isoproterenol in afflicted and control 4–5-month-old dogs differed significantly. In the anteroseptal region, it concentration-dependently prolonged APD in afflicted animals and had no effects on controls. As a result, at \( 10^{-8} \) and \( 10^{-7} \) mol/l isoproterenol, APD in afflicted dogs became significantly longer than the APD obtained prior to isoproterenol infusion. In the posterobasal region, isoproterenol had no significant action on afflicted dogs and decreased APD in controls.

By comparison, APD in neonates was significantly shorter than in afflicted and control dogs and the effects of isoproterenol qualitatively coincided with its actions on M cells of afflicted dogs: no significant change was seen at the posterior base but there was lengthening of APD in the anteroseptal region.

**EFFECTS OF 10^{-7} M ISOPROTERENOL ON ACTION POTENTIALS IN M CELLS**

Fig. 7. Representative experiments illustrating the effects of isoproterenol (\( 10^{-7} \) mol/l) on action potentials of M cells in transmural slices isolated from anteroseptal (AS) and posterobasal (PB) regions of left ventricle of mongrel adult and German shepherd dogs and mongrel puppies. CL=4000 ms, C, control.
Fig. 8. Concentration- and rate-dependent effects of isoproterenol on action potential duration at 90% repolarization (APD<sub>90</sub>) of M cells in transmural slabs isolated from the anteroseptal region of the left ventricle of mongrel and German shepherd controls and afflicted German shepherd dogs and mongrel puppies. Mean ± S.E.M. values are shown (n = 6 for mongrel, n = 7 for German shepherd and puppies; preparations with extrabeats were excluded from analysis). * P < 0.05 vs. control at the same CL, ** P < 0.05 vs. mongrel.

In the absence of isoproterenol there was no ectopic activity in M cell preparations at any CL. Isoproterenol induced premature depolarizations that appeared triggered by DAD. Their incidence depended on CL, ventricular region, type of animal, and isoproterenol concentration. Fig. 9 depicts the CL dependence of the incidence of triggered action potentials in the presence of 10<sup>-7</sup> mol/l isoproterenol (at lower concentrations there was no significant effect of isoproterenol). In the anteroseptal region, there were no triggered beats in controls and very few in neonates, whereas in afflicted dogs isoproterenol-induced triggered activity was observed at all CL from 300 to 4000 ms, attaining statistical significance at 500 and 1000 ms. In slabs from the posterobasal region, triggered activity was seen in all groups; however, its incidence did not reach significance. We then calculated the amplitude of those DADs that did not reach threshold potential (Fig. 10). It is of interest that DAD amplitude was significantly greater in both the anteroseptal and the posterobasal regions of afflicted dogs in comparison with controls and neonates. However, it is to be recalled that triggered activity was not significantly increased posterobasally (see Fig. 9) but only anteroseptally (Fig. 9). This suggests that the anomaly responsible for inducing DAD but not for triggering the arrhythmia is widespread in the myocardium of afflicted animals.

3.7. Studies of β-adrenergic receptors and adenylyl cyclase

Fig. 11 compares β-adrenergic receptor characteristics in tissues from the anteroseptal and posterobasal regions of six afflicted and four control German shepherds. In control dogs, β-adrenergic receptor density was lower in the anteroseptal than in the posterobasal region. In contrast, β-adrenergic receptor density was similar in both regions in afflicted dogs. Consequently, a significant difference in β-adrenergic receptor density was confined to the anteroseptal region; such that receptor density was higher in the afflicted than in the control dogs. In contrast, affinity for ICYP was similar in all preparations.

To assess the functional consequences of differences in β-adrenergic receptor density we next examined adenylyl cyclase activity in these membrane preparations (Table 2). Both basal and Gpp(NH)p-dependent adenylyl cyclase activity were higher in membranes from the anteroseptal and posterobasal regions of afflicted dogs compared with the comparable regions of control animals. Regional differences in basal or Gpp(NH)p-dependent adenylyl cyclase activity were not detected in either group of animals (Table 2). Activation via the β-adrenergic receptor (ISO+Gpp(NH)p) also was more robust in preparations from afflicted than from control animals as shown in Fig. 12. While isoproterenol induced a dose-dependent increase in adenylyl cyclase activity in all preparations, the activation was most pronounced in the anteroseptal region of afflicted animals, where the response was significantly greater than that observed either in the anteroseptal or posterobasal regions of control ventricles.

4. Discussion

The major findings of our study are: (1) the region of altered sympathetic innervation in a naturally occurring model of lethal arrhythmias is associated with altered
β-adrenergic signaling; (2) associated with the altered signaling pathway are an altered electrophysiologic responsiveness of the ventricular action potential to β-adrenergic agonist, resembling the response seen in neonatal animals; (3) there is the occurrence of β-agonist-induced DADs at both ventricular sites studied, but triggered activity is predominant at sites of altered innervation; (4) the CLs and coupling characteristics of the ventricular tachycardias in intact animals are consistent with DADs as the etiology; (5) the abnormalities in PR interval and the occurrence of sinus pauses suggest that supraventricular as well as ventricular abnormalities of innervation and/or receptor–effector coupling may occur; (6) we have confirmed that in Purkinje fibers, there is abnormal α-adrenergic responsiveness resulting in EAD and triggered activity, which can explain the pause-dependent ventricular tachycardia occurring in the majority of animals, but no abnormality of β-adrenergic response.

4.1. Relationship of abnormal innervation to receptor–effector coupling

Although we did not study innervation, we chose the anteroseptal left ventricle as a test site because the results of Dae et al. [4] identified this as a region of decreased sympathetic innervation. Moreover, alterations in sympathetic innervation would provide a logical determinant of the abnormal receptor–effector coupling that we found. Linkages between the sympathetic nervous system and the expression of clinical arrhythmias are well-recognized (e.g. [16–22]). The spectrum of arrhythmias is wide, and includes the torsades de pointes that characterizes the congenital long QT syndrome (LQTS) [15] and the ventricular tachycardias attributed to exercise and to catecholamine-dependence [21,22]. Although not as explicitly associated, reports relate subsets of sudden infant death syndrome (SIDS) to abnormal neural input
Amplitude of delayed afterdepolarizations (DAD) in presence of $10^{-7}$ M isoproterenol

![Graph showing Amplitude of delayed afterdepolarizations (DAD) in presence of $10^{-7}$ M isoproterenol]

M- normal mongrel, AS- arrhythmic shepherd, NM- neonatal mongrel, NS- normal shepherd

Fig. 10. Amplitude of delayed afterdepolarizations induced by one minute stimulation at BCL=250 ms in the presence of isoproterenol $10^{-7}$ mol/l. Slabs were isolated from anteroseptal (AS) and posterobasal (PB) regions of the left ventricle of mongrel and German shepherd controls, afflicted German shepherd and mongrel puppies. * $P<.05$ vs mongrel ($n=13$ for shepherd, $n=11$ for mongrel, $n=10$ for puppies).

[17,18,23] and the association of increased life expectancy with β-blocker therapy post myocardial infarction [24] has been attributed by some to prevention of arrhythmias, albeit without direct evidence. Complementing the clinical studies is a large basic literature incorporating both phenomenological and mechanistic reports of sympathetic modulation of rhythm and arrhythmias (e.g. [4,25–28]).

Despite the above, much uncertainty remains about the mechanisms whereby sympathetic input modifies cardiac rhythm and arrhythmias. For example, in congenital LQTS, where sympathetic abnormalities have been proposed [17] and ion channel abnormalities demonstrated [16,29–32], β-adrenergic blockade is of major antiarrhythmic benefit in many patients, while specific ion channel blockade shortens the QT interval in specific subsets of patients [33,34]. This implies that in a setting where a substrate (an ion channel) and a trigger mechanism (the sympathetic nervous system) are involved, the expression of any arrhythmia and its response to therapy have complex sets of determinants whose implications regarding mechanism may not be readily decipherable. Moreover, experimental animal data have shown that interrupting sympathetic innervation not only alters the response to adrenergic interventions [35], but also alters developmental evolution of specific ion currents [36].

Another factor contributing to the expression of sympathetically-related arrhythmias may be the age of an individual. This appears true of the German shepherd model, in that few spontaneous arrhythmias are seen during the first 3–4 months of life after which there are tachyarrhythmias and sudden death [3,37]. It was for this reason that we elected to study neonates, assuming that the electrophysiology of the immature ventricle that has sparse sympathetic innervation might provide some clues to the electrophysiologic characteristics of the afflicted German shepherds. Despite their relatively sparse sympathetic innervation, neonates do not have a propensity to tachyarrhythmia. The possible explanations of this include the numbers and the effector coupling of β-receptors in the neonate and the homogeneity of action potentials transmurally in the neonatal ventricle.

Yet another important determinant of the heterogeneity of sympathetic innervation in the German shepherds may be based on the pattern of development of innervation in the neonatal and young dog, which does not evolve uniformly. Rather, different regions of the ventricle are innervated at different rates [38]. One implication of this information is that the abnormal innervation and expression of arrhythmias in afflicted animals may result from a delay in development of innervation. However, whether
Fig. 12. Adenylyl cyclase activity in anteroseptal (AS) and posterobasal (PB) regions of control and afflicted German shepherds. Adenylyl cyclase activity was measured in the presence of 100 μmol/l Gpp(NH)p alone or 100 μmol/l Gpp(NH)p plus the indicated concentrations of isoproterenol according to Section 2. Results represent determinations from three control and six afflicted German shepherd preparations. * P<.05 vs. controls in same group. † P<.05 vs. all other groups.

called “catecholamine supersensitivity”. Hence, it is useful to consider together the findings of Dae et al., regarding innervation [4] and our own findings on the β-adrenergic receptor cascade. We focused on three components of the β-adrenergic receptor signaling cascade; the β-receptor, the adenylyl cyclase enzyme and the effector response. A unique finding is that β-adrenergic receptor density differs between anteroseptal and posterobasal regions in normal young German shepherd dogs. Such a difference is not unexpected given that innervation to various regions of the heart [38] and ion channels in different cardiac tissues [38] develop at different times. With respect to innervation, not only do sympathetic nerve branches to various regions within the LV and RV develop at different rates but both advance and regress developmentally until an adult pattern is attained [39]. Of further interest is that the heterogeneity in β-adrenergic receptor density seen in controls is lost in afflicted dogs i.e., receptor density in the anteroseptal region is greater in afflicted than in control animals and equals the density seen in the posterobasal regions of both groups of animals.

The consequences of the differences in β-adrenergic receptor density are complex and not readily decipherable. Certainly, generation of cAMP is the integrated output of the combined actions of the β-adrenergic receptor, the coupling G protein (a heterotrimer comprised of α subunits and βγ dimers which also are subjected to complex regulatory events) and the adenylyl cyclase enzyme. Moreover, there is recent evidence that the adenylyl cyclase enzyme itself may be the limiting component in the pathway linking β-adrenergic receptors to the generation of cAMP [40]. Thus, the robust increase in cAMP generation in both the anteroseptal and posterobasal regions of afflicted animals suggests that the abnormalities of the β-adrenergic receptor complex in the German shepherds

Fig. 11. β-Adrenergic receptor characteristics in control and afflicted German shepherds. B_max and K_d are depicted for anteroseptal (AS) and posterobasal (PB) regions. * P<.05 vs. control.
are not likely to be confined to the β-adrenergic receptor. In fact, as shown in Table 2, the control adenylyl cyclase activity was similar in anteroseptal and posterobasal regions although (see Fig. 11) there was a difference in β-receptor density between the two regions. Moreover, with adenylyl cyclase activation being most pronounced in the anteroseptal region of afflicted dogs, (with no difference in receptor density between the two regions) it appears that the evolution of the receptor and adenylyl cyclase activity occurs independently. This interesting situation has many possible bases: indeed, differences in β-adrenergic receptor subtype expression (β1 vs. β2, both of which can activate adenylyl cyclase) G protein subunit expression, and β-adrenergic receptor–G protein linkage are only a few of the numerous mechanisms that could influence cAMP formation in this tissue and which require further study. In addition, based on the effect of isoproterenol to prolong the action potential plateau of myocardium of afflicted animals, as well as preliminary results (from SFS) suggesting a greater effect of isoproterenol to increase intracellular Ca++ in myocardium from afflicted than normal animals, it is likely that the abnormal signal transduction in the afflicted group is associated with excess Ca++ that generates the occurrence of DADs. However, this hypothesis awaits definitive testing.

Another level of question here relates to the observation that cAMP elevation in the posterobasal region of the afflicted animals is nearly as great as that in the anteroseptal. On the one hand this explains the observation that significantly larger DAD occurred in the posterobasal region of afflicted animals than controls. This still leaves open the question of why DADs far more readily attained threshold and resulted in triggered activity in the anteroseptal than the posterobasal region. Also to be tested is the possibility that multiple abnormalities in myocardial substrate accompany the sympathetic and β-adrenergic changes seen. Specifically, the fact that differences in the PR interval and in sinus pauses occur in afflicted animals and controls suggests that neural and/or cardiac abnormalities occur supraventricularly as well as in the ventricle. Moreover, the occurrence of DADs but not triggered activity in posterobasal LV suggests a myocardial substrate abnormality in the absence of an essential trigger mechanism to bring it to threshold. These possibilities too, await testing.

4.2. Expression of ventricular tachycardia in vitro and in vivo

Our isolated tissue studies confirmed that α-agonist induces EAD and triggered activity in Purkinje fibers and demonstrated a new finding, the effect of β-agonist to induce DAD and triggered activity in midmyocardium of the anteroseptal region. Of importance here is the apparent segregation of each type of response: an α-adrenergic abnormality in the Purkinje system and a β-adrenergic abnormality in the myocardium. To our knowledge this type of sequestration of anomalies has not previously been reported, yet it offers an obvious explanation for the different types of arrhythmias seen clinically. EADs have been considered a likely cause of the pause-dependent ventricular tachycardia demonstrated in these animals; DAD are a likely cause of the tachycardia-induced ventricular tachycardias (see Fig. 2 regarding the CL dependency). As for the incidence of ventricular tachycardia, of 80 afflicted animals monitored by one of us (NSM), 83% had ventricular tachycardia that was pleomorphic and uniquely pause dependent, 11%, ventricular tachycardia that was monomorphic and not pause-dependent, and 6% had both types of tachycardia. Hence the two arrhythmogenic mechanisms may coexist in the same animal or be represented uniquely.

The other important association noted relates to the effects of isoproterenol on the myocardial (but not the Purkinje fiber) action potential. Specifically, in the myocardium, β-agonist elevated the plateau and accelerated repolarization in the anteroseptal and posterobasal regions of normal adult mongrel and shepherd dogs. This was in contrast to its lack of effect on or prolongation of repolarization in the neonate (in which sympathetic innervation is not yet developed.) From prior work we know that in neonatal epicardium, Ia is not demonstrable [39], Ik is unaffected by isoproterenol and Ica,L is increased by isoproterenol [35]. We have interpreted these results as indicating that in the normal, developing heart, the pathway for β-adrenergic receptor–effector coupling to Ica,L is intact prior to that for Ik.

A significant finding in the afflicted German shepherds is that plateau height is elevated by isoproterenol while action potential duration is prolonged, and most markedly in the anteroseptal zone. This is similar to isoproterenol’s effect on the neonatal canine heart, in which the β-adrenergic receptor effector pathway that activates Ik has not yet matured but there is a β-adrenergic effect to increase Ica,L [35]. The net result is an increase in APD. This increase in duration, which is manifested only at very long CLs is not, in itself, expected to be arrhythmogenic. Rather, we view it as another indicator of the abnormal receptor–effector coupling that occurs in these animals. Its occurrence implies a break in or a failure of development of the pathway linking β-receptors to Ik, but not that linking to Ica,L. This remains to be tested.

How does the above information relate to the clinical expression of arrhythmia in these animals? The arrhythmias are characterized by a multifactorial pattern of inheritance, in which no arrhythmia or QT abnormality is present at birth, but in which afflicted animals develop ventricular ectopy and tachycardia by 12–16 weeks of age [3]. There is a tendency for QT prolongation in afflicted animals (but no statistical significance to this). There are also notched T waves [41] and an anteroseptal reduction in
sympathetic innervation (shown using metaiodobenzylguanidine imaging and tyrosine hydroxylase staining) [4]. Lethal arrhythmias and death in some animals appear pause-dependent and are most marked when the animals are lying quietly or in REM sleep [42]. In others, the arrhythmia occurs when awake and/or excited. We may speculate that the EAD-induced mechanism determines the former clinical pattern and the DAD, the latter.

Obviously it is premature to associate the results of this or other studies in animal models to the evolution of arrhythmias in the developing heart. However, it is important to understand that although we may consider this only as a model for the human heart, it is a reality in the canine heart: i.e., a lethal condition determined by heredity. As such, the further unraveling of the pathways that contribute to arrhythmias and thus underlying mechanisms will offer insights into the complexities that also occur in man. One lesson, already apparent, is that a seemingly focal abnormality, the failure of sympathetic innervation to a portion of the myocardium, has broad “downstream” associations. Moreover, it is not yet clear if the failure of innervation is the cause of the myocardial anomalies seen or an accompaniment thereof. Understanding the complexity here may also shed light on the heterogeneity of responses in other disease entities in which ion channel and neural abnormalities have been associated, such as the congenital long QT syndrome. Specifically, we may begin to understand the expression of arrhythmias in some individuals whose QT intervals are normal and the failure to see arrhythmias in others in whom QT intervals are prolonged.

Acknowledgements

Supported by USPHS-NHLBI grant HL-28958; the Wild Wings Foundation; the INSERM, the Ministère de l’Agriculture (DGÉR), the Fondation de France and Royal Canin. The authors express their gratitude to Drs. Natalia Egorova and Wen Xiong as well as to Benjamin Wong, Ramya Mohan and Pascal Gervier for their assistance in performing certain of the studies, and to Ms. Eileen Franey for her careful attention to the preparation of the manuscript.

References


