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Dihydropyridine and beta adrenergic receptor binding in dogs with tachycardia-induced atrial fibrillation

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Abstract

Background: We have shown that rapid atrial activation, as occurs during atrial fibrillation (AF), reduces L-type Ca^{2+} current (I_{Ca}) and that this is the principal mechanism of the action potential duration and refractoriness changes that characterize tachycardia-induced atrial remodeling. The present study was designed to determine whether atrial tachycardia alters biochemical indices of the number of L-type Ca^{2+} channels and/or of the number and binding affinity of β-adrenergic receptors. **Methods:** In canine atrial sarcolemmal preparations, the number and binding affinity of dihydropyridine receptors were determined with the use of ^{3}H-nitrendipine and that of β-adrenergic receptors with ^{125}I-iodocyanopindolol. Results were obtained with preparations from dogs paced at 400/min for 1 (P1, $n=20$), 7 (P7, $n=9$), and 42 (P42, $n=9$) days, and compared with observations in sham-operated controls (P0, $n=14$). **Results:** Pacing reduced the B_{max} of dihydropyridine receptors, from 157±18 fmol/mg (P0) to 116±9 fmol/mg (P1, $P<0.05$), 100±14 fmol/mg (P7, $P<0.05$) and 94±9 fmol/mg (P42, $P<0.01$). The affinity of dihydropyridine receptors was unchanged, with the K_d averaging 711±102 pM, 656±74 pM, 633±155 pM and 585±92 pM in P0, P1, P7 and P42 dogs. Neither B_{max} nor K_d of β-adrenergic receptors was altered by rapid pacing. Values of B_{max} of dihydropyridine receptors correlated with atrial I_{Ca} current density ($r^2=0.95$) and ERP ($r^2=0.99$). **Conclusions:** Rapid atrial activation results in downregulation in the number of dihydropyridine receptors without altering the number or affinity of β-adrenergic receptors. The reductions in I_{Ca} that play an important role in the atrial electrical remodeling by which 'AF begets AF' appear to be due at least in part to a decrease in the number of L-type Ca^{2+} channels in cardiac cell membranes.

Keywords: Atrial fibrillation; Beta-adrenergic receptors; Cardiac electrophysiology; Electrical remodeling; L-type calcium channels

1. Introduction

Atrial fibrillation (AF) is the most common sustained cardiac arrhythmia encountered in clinical practice and, given its propensity to occur in the elderly, is likely to become more prevalent with the ageing of the population [1–3]. Several studies have demonstrated that atrial tachycardia promotes AF induction and maintenance in animal models, in association with reductions in effective refractory period (ERP) and ERP adaptation to rate [4–6]. The latter changes resemble findings in patients with enhanced susceptibility to AF [7]. We have obtained evidence suggesting that tachycardia-induced refractoriness alterations are caused by changes in action potential duration due to decreases in the density of L-type calcium current (I_{Ca}) [8].

I_{Ca} reductions could be due to a variety of mechanisms, including a decreased quantity of L-type Ca^{2+} channels or altered function of the channel such as might be produced by changes in regulation by β-adrenoceptors. β-Adrenergic stimulation activates voltage-dependent L-type Ca^{2+} channels through phosphorylation of the channel α-subunit [9].

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No published data are presently available regarding changes in dihydropyridine or β-adrenergic receptor properties in atria from animals rendered susceptible to AF by being exposed to chronic atrial tachycardia. The goal of the present study was to characterize the properties of dihydropyridine receptors (corresponding to L-type Ca^{2+} channels) and of β-adrenergic receptors in dogs subjected to chronic rapid atrial stimulation.

2. Methods

2.1. Pacemaker insertion and atrial pacing

Adult mongrel dogs of either sex (27.3±2.7 kg, n=52) were anesthetized with sodium pentobarbital (30 mg/kg i.v., followed by i.v. boluses of 4 mg/kg as needed). Artificial respiration was maintained via an endotracheal tube connected to a Harvard-type mechanical ventilator. Under sterile technique, a unipolar screw-in Medtronic J pacing lead (Medtronic) was inserted through the right jugular vein, and the distal end of the lead was fixed in the right atrial appendage under fluoroscopic guidance, as previously described [4]. Initial atrial capture was verified with the use of an external demand pacemaker (GBM 5880, Medtronic). The proximal end of the pacing lead was then connected to a custom-modified implantable Medtronic pacemaker unit (model 8084), which was inserted into a subcutaneous pocket in the neck.

Twenty-four hours were allowed for lead stabilization, and the pacemaker was then programmed to capture the atrium at 400/min (150-ms cycle length) with the use of 0.42-ms square-wave pulses at twice-threshold current. The atria were continuously stimulated at this rate for a period of 1 (group designated 'P1', n=20), 7 (group designated 'P7', n=9), or 42 (group designated 'P42', n=9) days. The surface ECG was verified after 24 h and then weekly to ensure continuous 1:1 atrial pacing. Rapidly-paced dogs were compared with sham dogs (P0, n=14), who were similarly instrumented but maintained without pacemaker activation.

2.2. Electrophysiological study

On study days, dogs were reanesthetized with morphine (2 mg/kg s.c.) and α-chloralose (120 mg/kg i.v. bolus, followed by a continuous infusion of 29.3 mg/kg h^{-1}) and ventilated with room air supplemented with oxygen. Respiratory parameters were adjusted to maintain physiological arterial blood gases (SaO_2>90%, pH 7.38 to 7.44). Body temperature was maintained at 37°C with a circulating-water temperature control system. Polyethylene catheters were inserted into the left femoral artery and both femoral veins and kept patent with heparinized saline solution (0.9%). The left femoral artery catheter was used to monitor arterial blood pressure and to obtain samples for blood gas determinations. The right and left femoral vein catheters were used to infuse saline and α-chloralose respectively. A median sternotomy was performed, and a pericardial cradle was created. Two bipolar Teflon-coated stainless steel electrodes were inserted into the right atrial appendage for recording and stimulation. A programmable stimulator (Digital Cardiovascular Instruments) was used to deliver 2-ms pulses at twice-threshold current. A P23 1D transducer (Statham Medical Instruments), electrophysiological amplifiers (Bloom), and a paper recorder (Astromed Model MT-95000) were used to record arterial blood pressure, six standard surface ECG leads, a right atrial electrogram, and stimulus artefacts.

2.3. Experimental protocol

In paced dogs, a surface ECG was first recorded to confirm maintenance of 1:1 atrial pacing at 400/min. The implanted pacemaker was then deactivated.

The ERP was measured with a train of 15 basic (S_1) stimuli followed by a premature (S_2) stimulus at an S_1S_2 interval which was decreased from 180 ms by 10-ms decrements, with the ERP defined as the longest S_1S_2 interval failing to produce a propagated response. The ERP was determined twice at a basic cycle length of 300 ms, and the mean of the ERP values obtained (always within 10 ms of each other) was used for data analysis.

After ERP was measured, AF was induced with a train of 10-Hz, 2-ms stimuli to the right atrial appendage at four times threshold current. AF was defined as a rapid (>450/min), irregular atrial rhythm with varying atrial electrogram morphology. AF was considered sustained if it persisted for >45 min, and was distinguished from atrial flutter on the basis of the irregularity of atrial electrogram morphology and frequency. AF was induced ten times if AF duration was <10 min in order to estimate the mean duration of AF. Two AF inductions were performed if episodes lasted between 10 and 45 min. If AF lasted for >45 min, and no briefer episodes occurred, mean AF duration was based on this episode and no further AF induction was attempted in order to avoid excessive prolongation of the experiment.

2.4. Sarcolemmal preparation

After electrophysiological parameters were obtained, both right and left atria of paced and sham dogs were excised, quick-frozen in liquid nitrogen, and preserved at −80°C until further use. Sarcolemmal preparations were performed as previously described with modifications [10]. All manipulations were performed at 4°C and all buffers were supplemented with the following: 1 mM 1,4-dithiothreitol, 5 mg/ml leupeptin, 10 mg/ml benzamidine, and 5 mg/ml soybean trypsin inhibitor. Typically, 10 to 20 g of atrial muscle was pulverized under liquid nitrogen, and further homogenized for 10 s in 55 ml of 50 mM Tris–

HCl, pH 7.8 containing 20 mM sodium pyrophosphate (buffer A) with a Brinkmann Polytron (PT-3000) at 23000 rpm. The homogenate was centrifuged at 1000*g* for 15 min (Sorvall RC-5C, SS-34 rotor). The supernatant was saved and the pellet was resuspended in 40 ml of buffer A, rehomogenized four times (30 s each) with a Polytron and then centrifuged as above. The supernatant was again saved and the pellet was reextracted two more times. The supernatants were pooled, filtered through four layers of gauze and centrifuged at 200 000*g* for 45 min (Beckman LE-80K, Ti 45 rotor). The resulting pellets were resuspended in 10 ml of 0.25 M sucrose with 10 mM Tris–HCl, pH 7.4 (buffer B) cushioned over 12 ml of 0.9 M sucrose, containing 10 mM Tris–HCl, pH 7.4. After centrifugation for 60 min at 120 000*g*, membranes at the 0.25/0.9 M sucrose interface were collected and diluted with three volumes of 10 mM Tris–HCl, pH 7.4 and centrifuged at 200 000*g* for 45 min. The sedimented membranes were resuspended in buffer B at a concentration of 2 to 4 mg/ml, quick-frozen in liquid nitrogen and stored at −80°C for subsequent use.

2.5. ^{3}H-nitrendipine and ^{125}I-iodocyanopindolol binding assays

Total dihydropyridine-receptor density was determined as previously described [10]. Membranes were sonicated 2×5 s to reduce nonspecific binding. Equilibrium ^{3}H-nitrendipine binding was carried out in a total volume of 0.5 ml at room temperature for 90 min in 10 mM Tris–HCl, pH 7.4 containing 1 mM $CaCl_2$ in addition to 300 μg of membrane proteins and 11 concentrations of ^{3}H-nitrendipine. Nonspecific binding was determined in the presence of 1 μM of unlabelled nitrendipine. Binding filters were pretreated for 1 h with 0.15% bovine serum albumin, 0.3% polyethyleneimine and Tris–HCl 25 mM, pH 7.4 to reduce nonspecific binding to filters. Membrane-bound ligand was separated from free ligand by rapid vacuum filtration with a 48-well Harvester (Brandel). The filters were washed with 10 ml of ice-cold 10 mM Tris–HCl, pH 7.4 and placed in liquid scintillation mini-vials along with 5 ml of fluid (Universol, ICN). Bound radioactivity was measured in a Beckman liquid scintillation spectrometer (LS 6500). Binding assays were performed in duplicate.

Total β-adrenoceptor density was determined as previously described [11,12]. For determination of the total number of β-adrenoceptors, 5 μg of membrane protein per assay was incubated with 12 different concentrations of ^{125}I-iodocyanopindolol in 75 mM Tris–HCl, pH 7.4 containing 12.5 mM $MgCl_2$ and 2 mM EDTA for 90 min at room temperature. Nonspecific binding was determined with 10 μM of the nonselective β-adrenoceptor antagonist alprenolol. Binding filters were pre-treated as for ^{3}H-nitrendipine studies and membrane bound ligand was separated as described above. The filters were washed with 10 ml of ice-cold 25 mM Tris–HCl, pH 7.4, placed in vials, and bound radioactivity was measured in an automatic gamma counter (Wallac 1470 Wizard). Binding assays were performed in triplicate. Protein concentration was quantified by the method of Bradford [13] with bovine serum albumin as the standard. Saturation binding data were analyzed using the program ALLFIT [14].

2.6. Statistical analysis

Statistical comparisons of multiple group means were performed by analysis of variance (ANOVA). Nonorthogonal decomposition using Dunnett's test for multiple comparisons with one control was used to study changes due to different rapid atrial pacing durations [15]. Bartlett's test was used to analyze the homogeneity of variances between sham and paced groups [16]. The Kruskal–Wallis test was used for nonparametric comparisons of unpaired measures. Linear regression was used to analyze the relationship between single dependent and independent variables. All average results are expressed as mean±SEM, and a two-tailed P value <0.05 was considered statistically significant.

3. Results

3.1. Effects of rapid atrial pacing on the duration of AF and atrial refractoriness

We evaluated the effect of atrial tachycardia on AF duration assessed by burst pacing. Table 1 shows that AF duration increased progressively as a result of atrial tachycardia ($P<0.0001$), a finding that is consistent with previously published results [4,6]. The number of dogs in which sustained AF was induced by burst pacing increased progressively, from none under control conditions to 8/9 (89%) of P42 dogs. AF occurred spontaneously within the end of the assigned period in 2/9 (22%) P7 dogs and 5/9 (56%) P42 dogs. Table 1 shows the effects of rapid atrial activation on ERP at a basic cycle length of 300 ms. Mean ERP was reduced by rapid pacing, with decreases already

Table 1
Effects of rapid atrial pacing on duration of AF and on atrial refractoriness[a]

Parameters	P0	P1	P7	P42
n	14	20	9	9
AF duration, s	9±2	628±327[b]	1257±305[c]	2402±316[d]
Sustained AF, *n* (%)	0 (0)	4 (20)	5 (56)[b]	8 (89)[d]
AF during RAP, *n* (%)	0 (0)	0 (0)	2 (22)	5 (56)[b]
ERP_{300}, ms	122±6	101±4[b]	93±4[c]	89±6[c]

[a] '*n*' indicates the number of dogs in each group; RAP: rapid atrial pacing; ERP_{300}: atrial effective refractory period at 300-ms basic cycle length. Values are mean±SEM.
[b] $P<0.05$.
[c] $P<0.01$.
[d] $P<0.0001$ vs. P0.

evident within 1 day of the onset of pacing and near minimal values observed after 7 days.

3.2. Dihydropyridine and β-receptor binding

Fig. 1 shows ^{3}H-nitrendipine binding assay results from a representative experiment in each group. Total bound radioactivity (filled circles) increased with ligand concentration and nonspecific binding (in the presence of 1 μM unlabeled nitrendipine, filled squares) was linearly related to labelled ligand concentration. Nonspecific binding averaged 26.4±3.9% of total binding at 2.5 nM ^{3}H-nitrendipine and did not exceed 50% of total binding. Subtraction of nonspecific from total binding yielded specific binding, which was saturable in all experiments. The representative results shown suggest a decrease in dihydropyridine-receptor binding capacity in paced dogs.

As shown by results from representative experiments in Fig. 2, ^{125}I-iodocyanopindolol binding was saturable and displaceable by the nonselective β-adrenoceptor antagonist alprenolol (10 μM). Nonspecific binding averaged 24.8± 4.0% of total binding at 100 pM ^{125}I-iodocyanopindolol and did not exceed 40% of total binding. The representative data shown do not suggest any change in β-adrenergic receptor density or affinity in paced dogs

Fig. 3 shows examples of Scatchard plots for dihydropyridine receptor binding (left) and β-adrenoceptor binding (right). Data points followed a linear relation. In neither case was the slope significantly altered, suggesting that K_d did not change. The horizontal axis intercept moved consistently to the left for dihydropyridine binding in paced dogs, suggesting a decrease in B_{max}. No such change was observed for β-adrenoceptors.

3.3. Changes in mean receptor binding parameters caused by atrial tachycardia

A progressive reduction in the maximum level of dihydropyridine binding was observed as a function of time in paced dogs. Mean data from 9–20 hearts in each group are shown in Fig. 4, and indicate a significant decrease in dihydropyridine-receptor densigy (B_{max}) in paced dogs, with a substantial decrease after 1 day of atrial tachycardia. In contrast to B_{max}, dihydropyridine-receptor binding affinity (K_d) did not differ significantly between paced and sham dogs. Mean data for β-adrenoceptors are

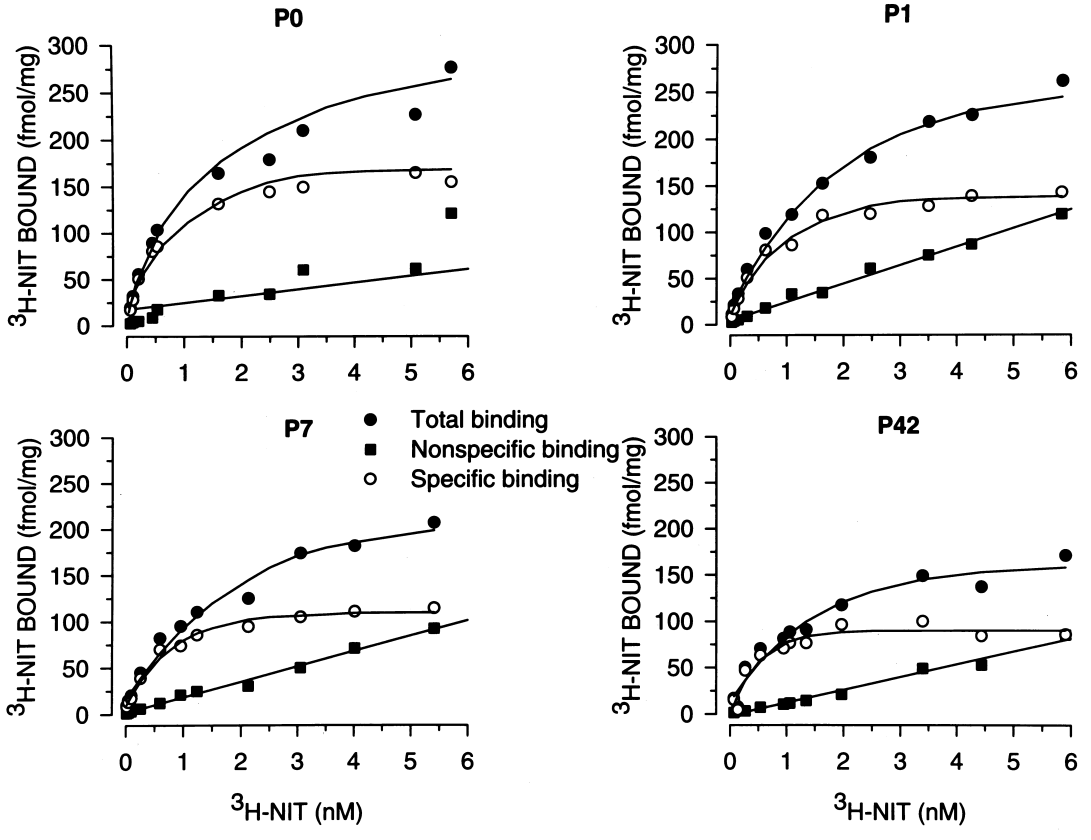


Fig. 1. Binding of ^{3}H-nitrendipine (^{3}H-NIT) to dihydropyridine-receptors in sarcolemmal preparations from unpaced (P0) and paced atria (P1, P7, P42). Representative saturation curves of ^{3}H-nitrendipine binding to sarcolemma showing total (solid circles), specific (open circles), and nonspecific (solid squares) binding are shown, along with experimental data points to which they were fitted. Dihydropyridine-receptor binding site densities (B_{max}) are 168 fmol/mg, 138 fmol/mg, 110 fmol/mg and 90 fmol/mg for the P0, P1, P7 and P42 hearts shown, respectively.

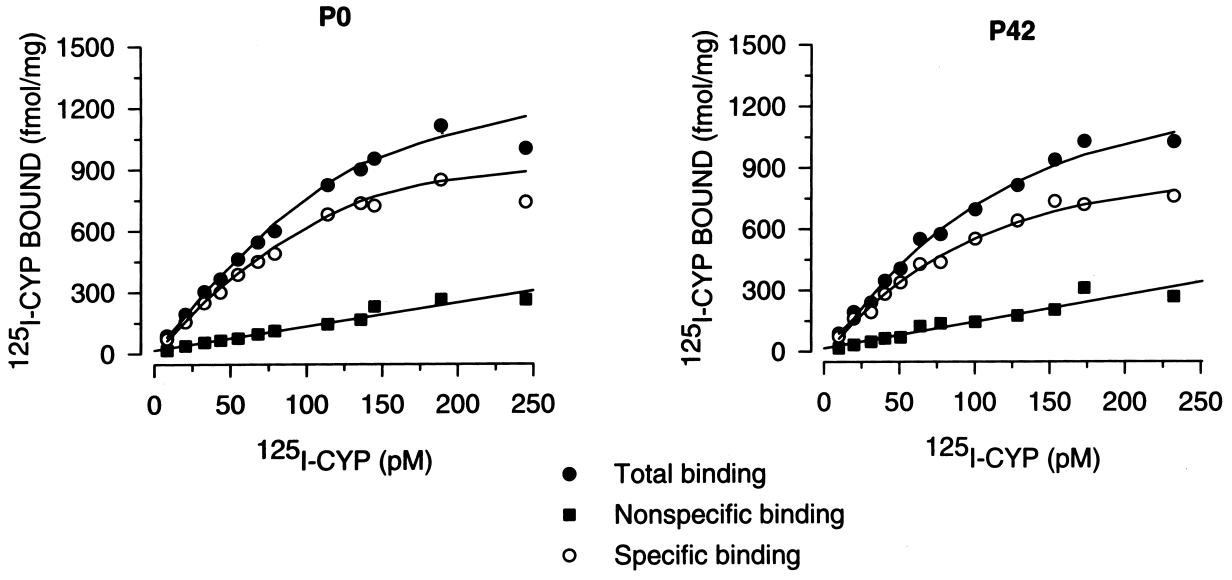


Fig. 2. Binding of ^{125}I-iodocyanopindolol (^{125}I-CYP) to β-adrenoceptors in sarcolemmal preparations from P0 and P42 dogs. Representative saturation curves of ^{125}I-odocyanopindolol binding to sarcolemma showing total (solid circles), specific (open circles), and nonspecific (solid squares) binding are shown, along with experimental data to which they were fitted. β-Adrenoceptor binding site densities (B_{max}) are 733 fmol/mg and 781 fmol/mg for P0 and P42 hearts shown, respectively.

shown in Fig. 5, and indicate that β-adrenoceptor density and binding affinity were unchanged by pacing.

3.4. Relations between changes in dihydropyridine-receptor density, I_{Ca} and atrial refractoriness

In order to relate the changes in dihydropyridine-receptor density to functional alterations, we plotted B_{max} of ^{3}H-nitrendipine binding against ERP in the same groups of dogs (Fig. 6A). There was a highly significant correlation (r^2=0.99) between dihydropyridine-receptor density and ERP. We also evaluated the relationship between previously measured values of L-type Ca^{2+} current density at +10 mV [8] and dihydropyridine-receptor density in the present experiments (Fig. 6B). A significant correlation was observed (r^2=0.95).

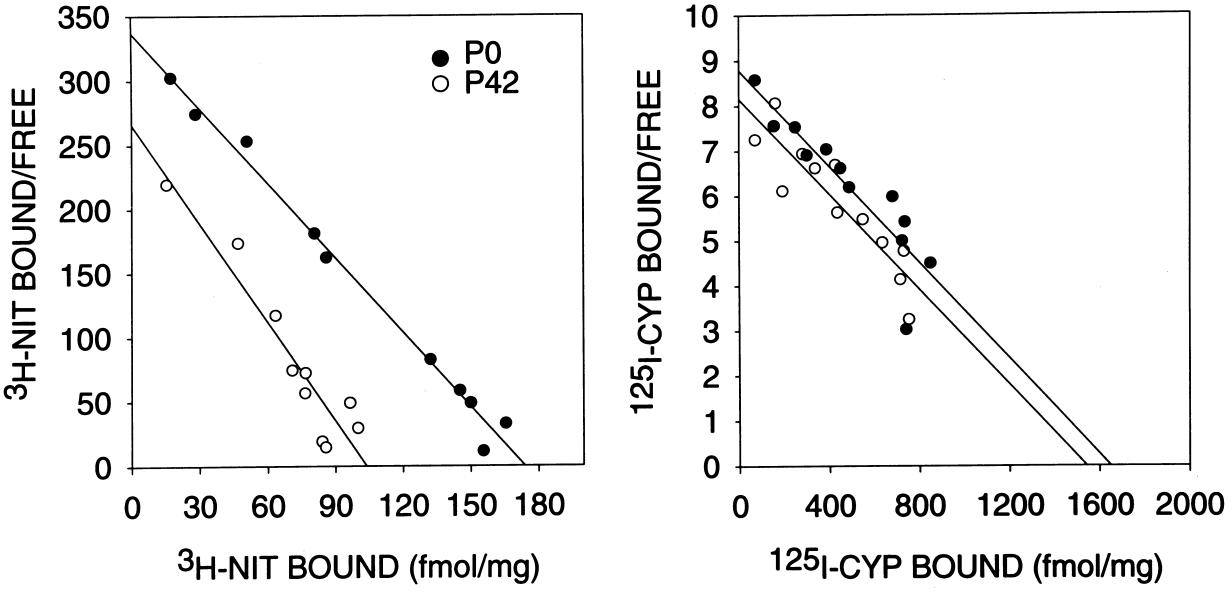
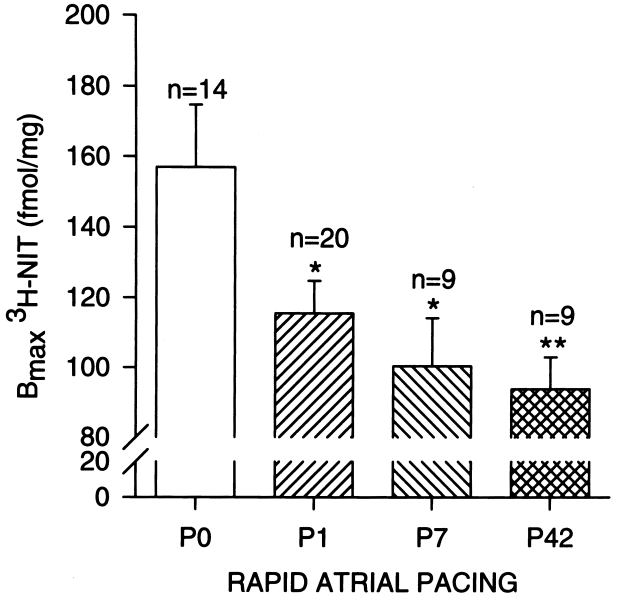


Fig. 3. Scatchard plots of ^{3}H-nitrendipine (left) and ^{125}I-iodocyanopindolol (right) binding. Least-squares regression lines to data are shown. For dihydropyridine binding, the data fits shown provided a B_{max} of 174 fmol/mg (P0) and 104 fmol/mg (P42), and K_d values of 515 pM (P0) and 390 pM (P42). For β-adrenoceptors, the values for B_{max} were 1648 (P0) and 1542 (P42) fmol/mg, whereas for K_d they were 188 (P0) and 189 (P42) pM.



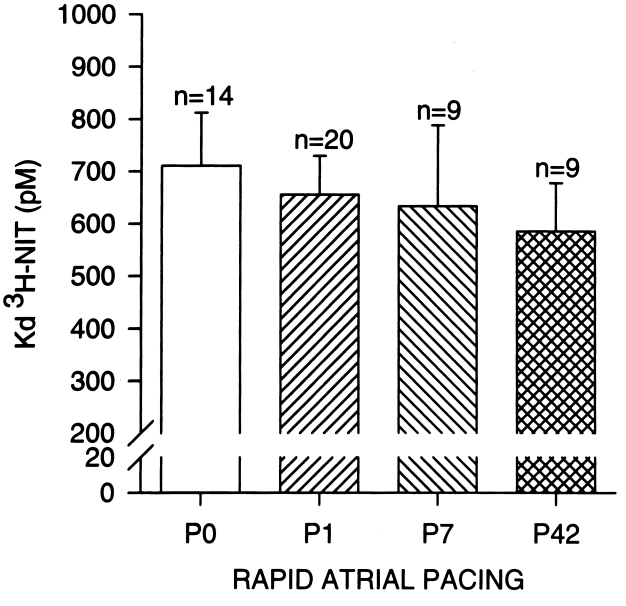
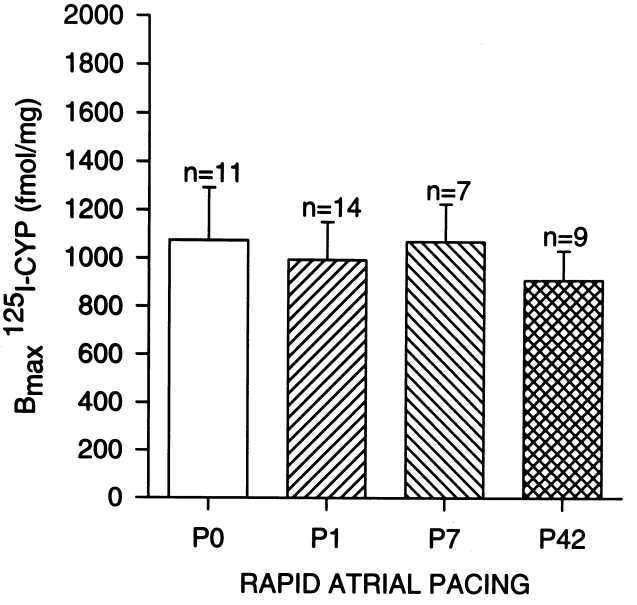


Fig. 4. Mean dihydropyridine-receptor density (B_{max}) and affinity (K_d) in atrial sarcolemmal preparations from P0 (n=14), P1 (n=20), P7 (n=9) and P42 (n=9) dogs. *P <0.05, **P <0.01 vs. P0.



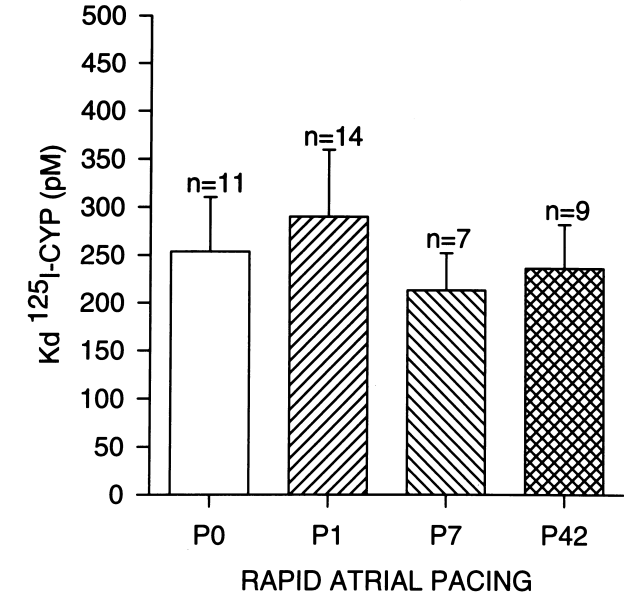


Fig. 5. Mean β-adrenoceptor density (B_{max}) and affinity (K_d) in atrial sarcolemmal preparations from P0 (n=11), P1 (n=14), P7 (n=7) and P42 (n=9) dogs. No significant changes were observed.

4. Discussion

We have demonstrated that rapid atrial pacing reduces the number of dihydropyridine-receptor sites, without altering their binding affinity. Neither specific binding capacity nor affinity of β-adrenergic receptors is altered. Changes in dihydropyridine-receptors parallel alterations in ERP and L-type Ca^{2+} current.

4.1. Altered dihydropyridine-receptor density in the tachycardia-induced model of AF

In animal models, rapid atrial activation results in a progressive and persistent increase in AF duration, with AF more prone to be sustained after a longer period of atrial tachycardia [4–6,17]. Alterations in AF duration are associated with a decrease in ERP and ERP adaptation to rate, changes often seen in patients with a tendency to exhibit atrial re-entrant arrhythmias and an increased vulnerability to AF induction [7]. We have provided electrophysiological evidence for a central role of decreases in L-type Ca^{2+} current in the ERP changes caused by atrial tachycardia [8], but the mechanisms by which I_{Ca} is decreased have not been clarified. The possibilities include a reduced number of channels in the sarcolemma, altered functional channel properties, and modified channel

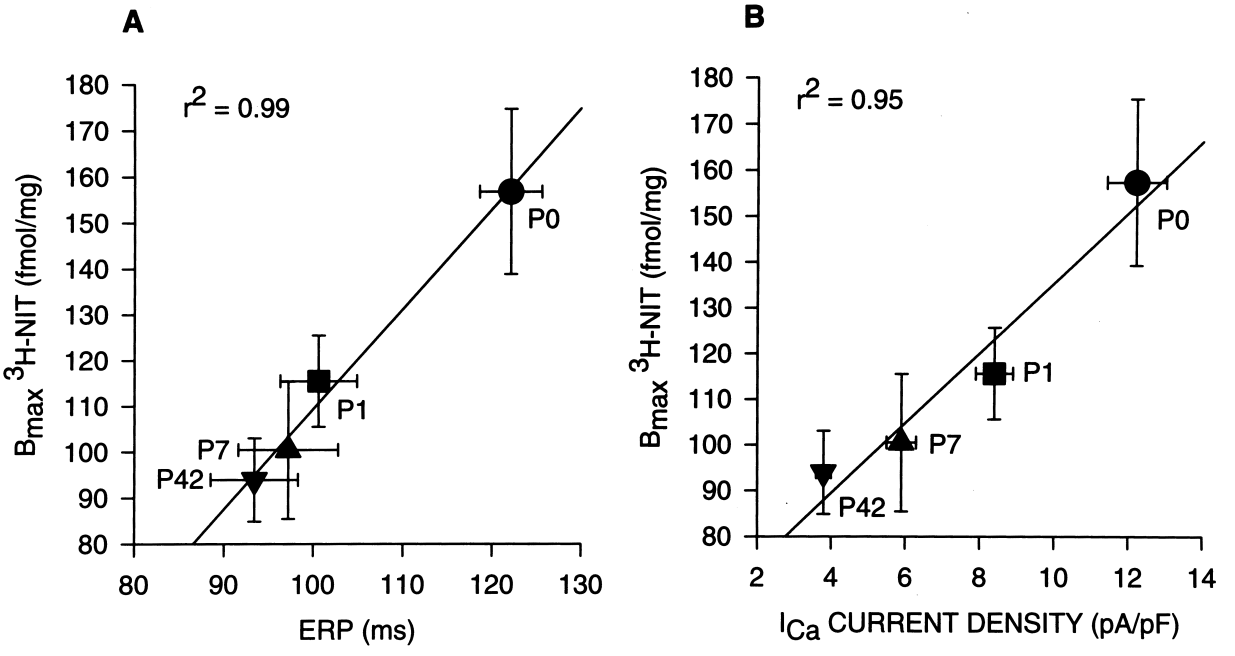


Fig. 6. A. Correlation between mean dihydropyridine-receptor binding and mean atrial ERP obtained at basic cycle length of 300 ms in the same dogs for each group. Results are mean±SEM for all measurements. B. Correlation between mean dihydropyridine-receptor binding from P0 (n=14), P1 (n=20), P7 (n=9) and P42 (n=9), and mean I_{Ca} current density, as measured previously [8] at +10 mV in 25 cells from five hearts in each group of dogs. (Note that the r^2 values are for the four data points for mean values shown in each graph).

regulation by guanine nucleotide binding proteins coupled to various transmembrane receptors. In the present study, we noted that rapid pacing reduced dihydropyridine-receptor density, suggesting a potentially important role for reduced L-type channel density. The correlation between dihydropyridine-receptor density and ERP is in agreement with the notion that a reduced number of Ca^{2+} channels leads to functional changes that contribute to the AF-perpetuating effects of atrial tachycardia. In combination with our previous observation of decreased concentration of the transcript encoding the α-subunits of the L-type Ca^{2+} channel [18], these observations suggest that a large part of the I_{Ca} decrease caused by sustained atrial tachycardia is due to transcriptional downregulation of the production of pore-forming α-subunits. An alternative explanation, especially for the decrease in dihydropyridine receptors after 24 h of rapid pacing, could be internalization of receptors.

A previous study reported that patients with end-stage heart failure had a reduction in the number of dihydropyridine-receptors in ventricular myocardium [19]. Atrial dihydropyridine receptors were not quantified. Other reports suggest increased or unchanged dihydropyridine-receptor density at various stages of left ventricular hypertrophy [20,21]. In experimental ventricular cardiomyopathy, cardiac dihydropyridine-receptors have also been found to be reduced in number with no change in ligand binding affinity [20]. Although congestive heart failure is associated with an increased incidence of AF, the mechanisms by which heart failure predispose to AF are quite different from those involved in the atrial tachycardia model. In particular, atrial ERP is not reduced [22], suggesting that I_{Ca} is not likely to be reduced to the same extent (if at all) compared with the rapid pacing model.

Previous studies have suggested that the voltage and time dependence of I_{Ca} are not altered in dogs subjected to rapid atrial pacing [8]. Thus, a change in channel function is unlikely to account for the I_{Ca} reduction observed in dogs with atrial tachycardia. The present study, which suggests that the number of L-type Ca^{2+} channels is reduced in rapidly paced dogs, provides an explanation for I_{Ca} reduction compatible with the biophysical observation of unaltered channel function.

4.2. Changes in β-adrenoceptor density in the tachycardia-induced model of AF

The α-subunit of cardiac L-type Ca^{2+} channels is phosphorylated by β-adrenoceptor stimulation [23], causing an increase in open probability of L-type Ca^{2+} channels and increasing I_{Ca}[24,25]. Marsh [26] has demonstrated in a myocyte culture system that β-adrenergic stimulation can produce concomitant down-regulation of β-adrenoceptors and dihydropyridine binding sites. In the present study, β-adrenoceptor number and affinity were unaltered by chronic atrial tachycardia, suggesting that the reductions in I_{Ca} caused by atrial tachycardia are not due to alterations in β-receptor function. In agreement with our findings, Wijffels et al. [27] provided evidence against a role of the β-adrenergic system in AF-induced atrial

remodeling. Chronic inhibition of I_{Ca} with calcium channel blockers did not alter the number of β-adrenergic receptors in atria from patients with congestive heart failure [28].

4.3. Limitations of our findings

In the present study, we noted a maximum decrease of about 40% in dihydropyridine receptors, compared to a maximum 70% decrease in I_{Ca} noted in dogs subjected to 6 weeks of atrial tachycardia [8]. The discrepancy could be due to technical differences (dihydropyridine receptors measured in sarcolemmal preparations of atrial tissue versus I_{Ca} in isolated myocytes), to a nonlinear relationship between dihydropyridine receptor number and the number of functional Ca^{2+} channels, or to the involvement of factors other than simply a change in the number of Ca^{2+} channels in the I_{Ca} reductions caused by chronic tachycardia. Intracellular Ca^{2+} handling is substantially altered in cells from dogs with atrial tachycardia [29] and intracellular Ca^{2+} is an important regulator of I_{Ca} function [30]. These changes in intracellular Ca^{2+} homeostasis could conceivably contribute to the I_{Ca} alterations that occur in rapidly-paced dogs.

The correlations shown in Fig. 5 point to the physiological relevance of the changes observed in dihydropyridine-receptor binding. On the other hand, it should be noted that the correlations were based on mean data in four groups (P0, P1, P7 and P42). It should also be noted that biochemical results were obtained from whole atrial samples, and that there can be significant regional variability in atrial ion channel expression [31] and in atrial tachycardia-induced remodeling [32].

5. Conclusions

Rapid atrial activation reduces the number of dihydropyridine binding sites in atrial sarcolemma of dogs. The reductions in dihydropyridine receptors correlate with ERP and I_{Ca} changes caused by rapid pacing, suggesting that decreases in Ca^{2+} channel number play a role in the ERP alterations which are a hallmark of atrial electrical remodeling caused by atrial tachycardia.

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