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INCREASED [^{3}H]NITRENDIPINE BINDING SITES IN RAT HEART DURING ADULT MATURATION AND AGING

SRISALA NAVARATNAM and JAGDISH C. KHATTER*
Departments of Internal Medicine and Pharmacology, University of Manitoba, Winnipeg, Manitoba, Canada

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Abstract—To study the effects of maturation and aging on calcium channels, we investigated the characteristics of binding of a radioligand, [^{3}H]nitrendipine, to relatively pure sarcolemmal membranes from 2-, 12- and 24-month-old Sprague–Dawley rat hearts. Specific binding of [^{3}H]nitrendipine was saturable, and the Scatchard analysis of the binding revealed a single class of binding sites. Binding of [^{3}H]nitrendipine to the membrane of 12-month-old-rats was 50–75% greater than to the membrane of 2-month-old young adult rats with no further changes in binding during aging from 12 to 24 months. The maximum number of dihydropyridine binding sites (B_{max}) was 70% higher in 12- and 24-month-old rat hearts (0.45 and 0.43 pmol/mg protein) than in 2-month-old rats (0.27 pmol/mg protein). The affinity for [^{3}H]nitrendipine binding, on the other hand, was similar in all three age groups (K_D values of 0.27, 0.31 and 0.29 nM in 2-, 12- and 24-month-old rats, respectively, at 25°). Membranes of all three age groups showed a similar degree of enrichment in sarcolemmal marker enzymes, indicating that the difference in membrane purity was not a contributing factor to the observed increase in density. Furthermore, increased binding of [^{3}H]nitrendipine to the membranes of older rat hearts was observed throughout the purification scheme. Since [^{3}H]nitrendipine binding sites are considered to be specific sites for voltage-gated Ca^{2+} channels of the sarcolemma, it is concluded that the density of these channels in the myocardium increases during adult maturation and is maintained through senescence.

Alterations in cardiac function with aging have been reported in humans and in various animal models [1–6]. Many of the animal studies have employed the rat as the animal model. In the rat, changes in myocardial function and metabolism are observed as early as during adult maturation [5–7], and progress through senescence [3, 4, 7]. Thus, the duration of isometric contraction of rat heart papillary muscle is prolonged during maturation and senescence [3–6]. The duration of contraction is determined by the duration of Ca^{2+} transient, which in turn is determined by the rate of Ca^{2+} influx via sarcolemma and the rate of Ca^{2+} release and uptake by sarcoplasmic reticulum (SR). The rate of myofilament shortening, which is determined by the rate of ATP hydrolysis by the myofilament, also contributes significantly in the determination of contraction duration. Studies in rats have indicated a progressive decline in myosin ATPase activity with aging of the animal due to the change in isomyosin composition [8, 9]. However, the prolongation of contraction duration has also been observed in humans, guinea pigs, and in dogs [1, 10, 11], where the isomyosin transformation is highly unlikely [12]. This suggests that alteration in Ca^{2+} metabolism is the major contributor to the prolonged contraction duration.

Studies in isolated SR membrane vesicles have revealed that the Ca^{2+} uptake in these vesicles progressively decreases with aging, without any alteration in the activity of Ca^{2+}-ATPase [7, 13]. These studies, however, did not focus on the state of the Ca^{2+} release sites of the SR and their contribution to the observed decline in Ca^{2+} uptake. In addition, in spite of these observed changes in Ca^{2+} uptake by the SR, the tension development remains the same during maturation and senescence, indicating a possible compensatory increase in Ca^{2+} influx through the sarcolemma. Furthermore, studies have also indicated a prolongation of action-potential duration during maturation and senescence with greater amplitude of action potential in the senescent myocardium [3, 5, 6]. Since the repolarization is initiated by the activation of K^+ channels and inactivation of voltage-dependent Ca^{2+} channels [14, 15], either the delayed activation of the K^+ channel or the prolonged activation of the Ca^{2+} channel may cause a delay in the repolarization and thus may prolong the action-potential duration [16].

In this study, we investigated the possibility that the myocardial voltage-gated Ca^{2+} channels may be modified during the process of maturation and senescence. Myocardial calcium channels possess receptor sites for various groups of calcium channel ligands [17]. Tritiated congeners of these ligands have been used to study their receptor sites [18–20]. To test our hypothesis, we carried out radioligand binding studies using a tritiated calcium channel antagonist, nitrendipine, in relatively pure sarcolemmal membrane from 2-, 12- and 24-month-old rat hearts.

MATERIALS AND METHODS

Male Sprague–Dawley rats of 2, 12 and 24 months of age (Harlan Industries, USA) were used in the

* Correspondence: Dr. J. C. Khatter, F330, Cardiology, Health Sciences Centre, 700 William Ave., Winnipeg, Manitoba, Canada R3E 0Z3.

present investigation. These rats reach sexual maturity around 2–3 months of age, adult maturation at 6–12 months, and senescence at around 24 months.

Isolation of sarcolemmal membrane. Relatively pure sarcolemmal membrane vesicles were prepared as described by us earlier [21]. Briefly, the left ventricles of the rat heart were homogenized in 0.6 M sucrose, 10 mM imidazole/HCl (pH 7) and centrifuged at 10,000 *g* for 20 min. The supernatant was diluted (2-fold) with 160 mM KCl/20 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS) (pH 7.4) and centrifuged at 96,000 *g* for 60 min. The pellet was resuspended in 2 mL of KCl/MOPS and layered over 15 mL of 30% sucrose solution containing 0.3 M KCl, 50 mM sodium pyrophosphate and 0.1 M Tris–HCl (pH 8.3) and centrifuged at 95,000 *g* for 90 min. The white band at the sample sucrose interface was recovered, diluted with 3 vol. of KCl/MOPS, and centrifuged at 100,000 *g* for 30 min. The pellet was resuspended in Tris–HCl to a final protein concentration of approximately 1 mg/mL. The protein concentration was determined by the method of Lowry *et al.* [22].

Specific [^{3}H]nitrendipine binding. About 80–100 μg of the sarcolemmal fraction, isolated by the above described procedure, was incubated in the medium containing 50 mM Tris–HCl buffer (pH 7.4). After 10 min of preincubation, the reaction was started by the addition of a given concentration (0.05 to 1.0 nM) of [^{3}H]nitrendipine and incubated for 60 min at 25°. Non-specific binding was determined in the presence of 0.1 μM non-labeled nitrendipine in the medium and was subtracted from the total [^{3}H]nitrendipine bound to obtain specific binding. At the end of the incubation period, the samples were immediately filtered through GF/B Whatman (Fisher Scientific) filters, using a constant vacuum suction system. The filters were washed three times with 3 mL of ice-cold 50 mM Tris–HCl buffer (pH 7.4), dried under vacuum, and then placed in 10 mL of scintillation fluid (Fisher Scientific) and counted using a Beckman LS8100 liquid scintillation counter. Correction for quenching was performed by the external channel ratio method.

Assays for sarcolemmal and subcellular marker enzymes. The relative purity of the final sarcolemmal membrane fractions was judged by various marker enzyme activities in homogenate, microsomes and sarcolemmal membrane. The sarcolemmal enzymes such as patent and ouabain-sensitive Na^+,K^+-ATPase and *p*-nitrophenol phosphatase (pNPPase) were assayed, in order to evaluate the distribution of sarcolemmal vesicles. These enzyme assays were carried out as previously described [23, 24].

The activities of mitochondrial ATPases are inhibited by sodium azide. By the addition of 5 mM sodium azide to the assay medium, the contribution of mitochondrial ATPase to the total ATPase activity was determined as described elsewhere [23] and was used as an estimation of mitochondrial contamination. K^+-EDTA ATPase is a good *in vitro* measure of myofibrillar ATPase which was assayed by a previously described method [25] and was used as a measure of myofibrillar contamination. The Ca^{2+}-stimulated (with low Ca^{2+}) ATPases, located in myofibrils and sarcoplasmic reticulum, were used as markers for the sarcoplasmic reticulum as well as myofibrils [26, 27]. The assay for these enzymes was carried out by a previously described method [27].

Data analysis. Radioligand binding was analyzed by Scatchard plot and computer analysis (radioligand binding analysis program by Munson and Rodbard as modified by McPherson [28]). Significant differences in slope (K_D) and B_{max} values were accepted at the confidence level of $P < 0.05$, using Student's *t*-test.

Chemicals. [^{3}H]Nitrendipine (sp. act. 71 Ci/mmol) was obtained from New England Nuclear and its purity was >97%. Non-labeled nitrendipine was supplied by Miles Pharmaceuticals, USA, courtesy of Dr. Alexander Scriabine.

RESULTS

Animal condition. The changes in body weight and heart weight of rats with advancing age are illustrated in Fig. 1. It can be seen that body weight continued to increase at a rapid pace up to 1 year of age before levelling off and remaining fairly constant thereafter. Similar changes in heart weight in relation to animal age were observed such that the ratio of heart weight to total body weight remained fairly constant in these age ranges.

[^{3}H]Nitrendipine binding. Figure 2 illustrates the specific binding of [^{3}H]nitrendipine to the sarcolemmal membrane of 2-, 12- and 24-month-old rat hearts as a function of its concentration. Binding of [^{3}H]nitrendipine was saturable, and occurred at a concentration of 0.54 nM in 2-month-old rat heart membranes. Scatchard plot analysis of the specific binding data (Fig. 3) yielded a monophasic plot over the concentration range tested (0.05 to 1 nM), indicating the existence of a single class of specific binding sites for [^{3}H]nitrendipine. Binding of [^{3}H]nitrendipine to the membrane protein of 12-month-old rats was almost 50–75% greater than in 2-month-old rat hearts, and there was no further increase in binding observed in 24-month-old rats (Fig. 2). Scatchard plots of specific binding data of 2-, 12- and 24-month-old rats are shown in Fig. 3. The maximum number of binding sites in the membranes of 12- and of 24-month-old rat hearts was 70% greater than that in 2-month-old rats (Table 1). There was virtually no change in the equilibrium dissociation constant (K_D), indicating that the affinity to bind [^{3}H]nitrendipine was similar in all three age groups. The maximum number of binding sites in 24-month-old rat heart sarcolemmal membrane fraction did not differ significantly from that of the 12-month-old rat.

Figure 4 illustrates the specific [^{3}H]nitrendipine binding to homogenate, microsomes and final sarcolemmal membrane of 2- and 12-month-old rat hearts, in the presence of 0.6 nM [^{3}H]nitrendipine. The binding of [^{3}H]nitrendipine to the sarcolemmal membrane fraction was 7-fold greater than that to homogenate, which was similar in both age groups. Furthermore, the specific binding in all three membrane fractions of 12-month-old rat heart was greater by 50% as compared to the respective membrane fractions of 2-month-old rat heart. Non-specific binding of [^{3}H]nitrendipine to the

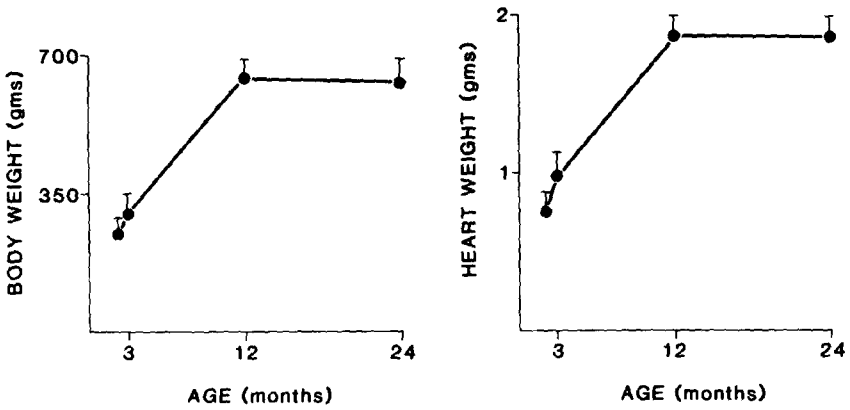

Fig. 1. Age-dependent alterations in body weight and heart weight of rats.

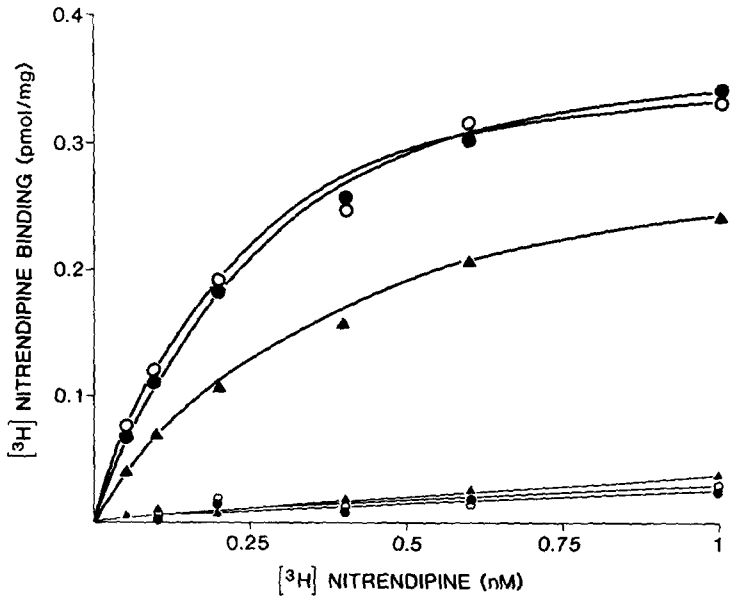

Fig. 2. [^{3}H]Nitrendipine binding to cardiac sarcolemmal membrane. Binding of [^{3}H]nitrendipine was carried out in membrane of 2- (▲), 12- (●) and 24- (○) month-old rats hearts. The level of binding was determined after 60 min of incubation at 25° in Tris–HCl (pH 7.4) in the presence of increasing amounts of [^{3}H]nitrendipine (0.05 to 1 nM). The results depicted in the figure represent the mean of duplicate measurements in a typical experiment. Experiments were carried out in five different membrane preparations, from 2-, 12-, and 24-month-old rat hearts. Non-specific binding of [^{3}H]nitrendipine was 15% of total binding at a concentration of 1 nM.

sarcolemmal membrane was similar in all three age groups of animals (Fig. 2).

Purity of the sarcolemmal membrane isolated from 2-, 12- and 24-month-old rat heart. To further ascertain whether the difference in membrane purity was a contributing factor to the observed increased density of [^{3}H]nitrendipine binding sites, sarcolemmal and intracellular marker enzymes were assayed. The recovery of sarcolemmal membrane protein was virtually the same in all three age groups (0.55 ± 0.1 mg protein/g tissue). As shown in Table 2, patent Na^+,K^+-ATPase and ouabain-sensitive Na^+,K^+-ATPase in purified sarcolemmal membrane did not differ significantly between 2- and 12-month-old rat hearts. The activity of Na^+,K^+-ATPase in 24-month-old rat heart, however, was significantly lower ($P < 0.05$) than that in 2- and 12-month-old rat hearts. These alterations in Na^+,K^+-ATPase activity have been demonstrated to be due to aging [29, 30]. The activity of pNPPase was also virtually identical in 2- and 12-month-old rats although somewhat lower activity was observed in 24-month-old rat hearts. The final membrane fractions of all three age groups of animals contained 8–12% of azide-sensitive ATPase, suggesting a small but equal contamination of mitochondria. The activities of

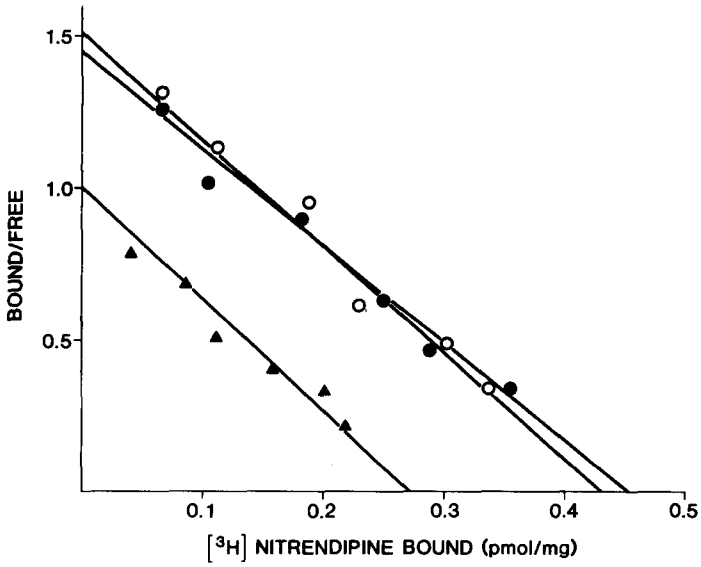

Fig. 3. Scatchard plots of specific [^{3}H]nitrendipine binding. The [^{3}H]nitrendipine binding was carried out using cardiac sarcolemma of 2- (▲), 12- (●), and 24- (○) month-old rats. Values are the means of duplicate measurements in a typical experiment. Lines were determined by linear regression analysis (r values of 2, 12- and 24-month-old membranes were 0.95, 0.88 and 0.89 respectively). The equilibrium dissociation constant (K_D) was calculated from the negative reciprocal of the slope, and the density of sites (B_{max}) was determined from the intercept of the lines with the abscissa; the values are given in Table 1.

Table 1. Binding constants of [^{3}H]nitrendipine for sarcolemmal membrane of 2-, 12- and 24-month-old rat hearts

Age (months)	B_{max} (pmol/mg protein)	K_D(nM)
2	0.27 ± 0.03	0.27 ± 0.04
12	0.45 ± 0.06*	0.31 ± 0.06
24	0.43 ± 0.05*	0.29 ± 0.07

Values are means ± SEM of experiments carried out in five different membrane preparations of 2-, 12- and 24-month-old rats.

* Significantly different from the appropriate value of 2-month-old rats (Student's t-test, two-tailed, $P < 0.05$). No significant difference was observed between the B_{max} values of 24- and 12-month-old rats.

either Ca^{2+}-stimulated ATPase or K^+-EDTA ATPase were not detectable in the final membrane fraction.

DISCUSSION

[^{3}H]Nitrendipine has been used to study the dihydropyridine binding sites of voltage-gated calcium channels in various membrane and tissue preparations [31–34]. In the present study, binding of [^{3}H]nitrendipine to purified sarcolemmal membrane fraction demonstrated an interaction to a single class of saturable binding sites, which is in agreement with earlier observations [31–33]. The K_D value of 0.27 nM obtained in this study for [^{3}H]Nitrendipine binding to the membrane from 2-month-old rat heart is similar to the K_D values reported earlier [31–34]. In general, the maximum (B_{max}) number of binding sites observed in studies reported earlier [29–33], with the exception of the study by Vaghy *et al.* in dog cardiac sarcolemma [33], was lower (0.062 to 0.187 pmol/mg) than what we observed in the present study. These differences in B_{max} values were probably due to differences in experimental conditions and/or different degrees of membrane purity. The differences in animal species may also be a contributing factor.

The number of dihydropyridine ([^{3}H]nitrendipine) binding sites in the sarcolemmal membrane was increased by 70% during adult maturation of rat from 2 to 12 months; this level was maintained during further aging from 12 to 24 months. The affinity of these sites for [^{3}H]nitrendipine remained unaltered during adult maturation and aging. Binding of [^{3}H]nitrendipine was also carried out in homogenates and microsomes of 2- and 12-month-old rat hearts. The enrichment of [^{3}H]nitrendipine binding sites in the final sarcolemmal membrane fraction was 7-fold, which was equal in both age groups. Furthermore, binding of [^{3}H]nitrendipine was increased to a similar extent in all three membrane fractions of 12-month-old rat heart, indicating that the observed increase in density of dihydropyridine binding sites in 12- and 24-month-old rat hearts reflects a true age-related alteration. In our experiments, the yield of the sarcolemmal membranes was virtually the same in all age groups. The enrichment of sarcolemmal marker enzymes

Table 2. Sarcolemmal and subcellular marker enzyme activities in membrane fractions isolated from 2-, 12- and 24-month-old rat hearts

	Na^+,K^+-ATPase (μmol/mg/hr)	Na^+,K^+-ATPase + 1 μM ouabain (μmol/mg/hr)	pNPPase (μmol/mg/hr)	K^+-EDTA ATPase (μmol/mg/hr)	Ca^{2+}-stimulated ATPase (μmol/mg/hr)
Homogenate					
2 months	3.2 ± 0.4	NC*	112 ± 6	16.9 ± 5	2.2 ± 0.8
12 months	3.8 ± 0.6	NC	106 ± 12	18.2 ± 6	2.1 ± 1
24 months	1.8 ± 0.6†	NC	93 ± 4	20.0 ± 6	3.14 ± 1
Crude membrane					
2 months	15.2 ± 1.7	6.3 ± 2	398 ± 9	1.4 ± 0.8	1.3 ± 0.7
12 months	15.6 ± 2	5.8 ± 3	390 ± 9	1.2 ± 0.7	1.6 ± 0.9
24 months	7.2 ± 4†	2.1 ± 1	360 ± 12	1.8 ± 0.8	1.8 ± 0.7
Final SL membrane					
2 months	31.6 ± 3	18.9 ± 0.9	682 ± 18	ND‡	ND
12 months	34.7 ± 4	21.8 ± 3	664 ± 17	ND	ND
24 months	17.4 ± 3†	9 ± 0.4	600 ± 6	ND	ND
Purification factor					
2 months	9.8	—	6.1	0	0
12 months	9.1	—	6.3	0	0
24 months	9.5	—	6.5	0	0

Enzyme activities were assayed in all three membrane fractions (homogenate, crude membrane and final sarcolemmal membrane) which were used for the [³H]nitrendipine binding studies. The methodological details are provided under Materials and Methods. Values are means ± SEM of 4–5 experiments carried out in independent membrane preparations. The purification factor indicates the enzyme activity in the final sarcolemmal membrane/homogenate.

† Significantly different when compared to the appropriate value of 2- and 12-month-old rat hearts (Student's *t*-test, two-tailed, $P < 0.05$).

‡ Not detected.

* Not carried out.

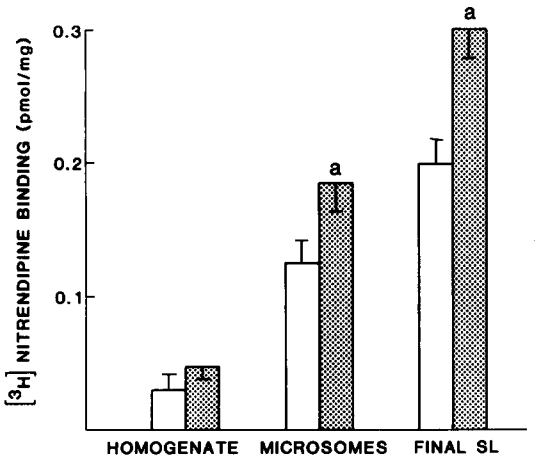

Fig. 4. Specific [^{3}H]nitrendipine binding to homogenate, microsomes and final sarcolemmal (SL) membrane. The binding was carried out in membranes of 2 (□) and 12-(□) month-old rat hearts in the presence of 0.6 nM [^{3}H]nitrendipine (concentration at which saturation of binding occurred in the final SL membrane). The homogenates were diluted with buffer (KCL/MOPS: pH 7.4) to obtain a final protein concentration of 2.5 to 3 mg/mL and the binding of [^{3}H]nitrendipine was carried out using 250–300 μg of protein as described under Materials and Methods. The membrane fraction obtained after the first centrifugation at 96,000 *g* is represented as microsomes, and the binding of [^{3}H]nitrendipine was determined in a similar manner to that for the final SL membrane. Values are the means of three experiments carried out in three different membrane preparations of homogenates and microsomes and five different preparations of final SL membrane from 2- and 12-month-old rat hearts. Key: (a) significantly ($P < 0.05$) different from 2-month-old.

Na^+,K^+-ATPase and pNPPase was also essentially similar in 2- and 12-month-old rats, further demonstrating that the purity of sarcolemmal vesicles was not different in the two age groups. The reduction of Na^+,K^+-ATPase activity during senescence has been reported earlier by us and others [29, 30]. The purification factor of pNPPase was lower than that of Na^+,K^+-ATPase in membranes of all three age groups. However, it should be pointed out that exact parallelism is not always observed during enrichment of various sarcolemmal marker enzymes [35]. Our final membrane fraction has minor contamination from mitochondria, which was equal in all three age groups and had no contamination either from SR or myofibrils. The findings mentioned above clearly indicate that the purity of the membrane was not a significant factor in the observed increase in [^{3}H]nitrendipine binding in 12- and 24-month-old rats. Since the [^{3}H]nitrendipine receptor sites are likely to represent voltage-gated calcium channels, the present observation would mean that the density of myocardial calcium channels increases during adult maturation and is maintained through senescence. In agreement with our observations, Battaini *et al.* [36] have also observed an age-dependent increase in [^{3}H]verapamil binding to rat cortical membrane, suggesting that the alteration in Ca^{2+} channels with aging may be a general phenomenon.

Myocardial calcium channels mediate Ca^{2+} influx and contribute to the plateau phase of action potential, during which they undergo cycles of brief openings and closings [37–38]. With an increase in the number of these channels in older rat hearts, the total time of Ca^{2+} channel activation may be prolonged. This, in turn, may delay the repolarization, resulting in prolonged action-potential duration as observed in 12- and 24-month-old rats [3, 5, 6]. Prolongation of action-potential duration and increased duration of Ca^{2+} influx through sarcolemma will also be reflected on the duration of myoplasmic Ca^{2+} transient. Further, the contraction and relaxation of myofibrils are dependent on myoplasmic Ca^{2+} fluctuation during excitation. Hence, the increased duration of myoplasmic Ca^{2+} transient may also prolong the duration of contraction in older rat hearts. Studies have also indicated a progressive increase in duration of contraction from 12 to 24 months [4], which may be influenced by the changes in other factors such as reduction in Ca^{2+} uptake by SR and reduced Na^+-Ca^{2+} exchange activity [7, 40].

If the number of voltage-gated calcium channels increases with advancing age, one would also expect to find an altered response to agents that influence the influx of Ca^{2+} via these channels. In fact, Rosen *et al.* [41] observed an age-dependent increase in sensitivity to the calcium antagonist AH 2666 as measured by action-potential parameters in canine Purkinje fibers of 5-year-old dogs as compared to 1.5 year olds. In our laboratory, we have demonstrated a substantially higher sensitivity of 12-month-old rat hearts to BAY K 8644 when compared with those of 2 month olds*. Buhler *et al.* [42] have also demonstrated that the response of hypertensives to calcium antagonist therapy becomes progressively greater as the patients get older. Similar to aging, alterations in mechanical function of the myocardium have also been demonstrated with chronic hypertension [43]. Moreover, the activities of voltage-gated calcium channels of the myocardium and vascular smooth muscle were also found to be greater in chronic hypertension [44, 45], suggesting that the modification of myocardial voltage-gated calcium channels is, in fact, a primary contributor to the altered mechanical properties of the myocardium. This would support the fact that the observed increase in the number of Ca^{2+} channels with adult maturation is most probably associated with altered function, which may be of therapeutic importance. Future studies are necessary, however, to directly demonstrate the increase in Ca^{2+} influx/Ca^{2+} current via voltage-gated Ca^{2+} channels.

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