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Research Article

Previous studies have demonstrated that the ability of beta-adrenergic receptor (beta AR) stimulation to increase cardiac contractility declines with aging. In the present study, the control mechanisms of excitation-contraction (EC) coupling, including calcium current (ICa), cytosolic Ca2+ (Cai2+) transient and contraction in response to beta AR stimulation were investigated in ventricular myocytes isolated from rat hearts of a broad age range (2, 6-8, and 24 mo). While the baseline contractile performance and the Cai2+ transient did not differ markedly among cells from hearts of all age groups, the responses of the Cai2+ transient and contraction to beta-adrenergic stimulation by norepinephrine (NE) diminished with aging: the threshold concentration and the ED50 increased in rank order with aging; the maximum responses of contraction and Cai2+ transient decreased with aging. Furthermore, the efficacy of beta AR stimulation to increase ICa was significantly reduced with aging, and the diminished responses of the contraction and Cai2+ transient amplitudes to NE were proportional to the reductions in the ICa response. These findings suggest that the observed age-associated reduction in beta AR modulation of the cardiac contraction is, in part at least, due to a deficit in modulation of Cai2+, particularly the activity of L-type calcium channels.

Age-associated Changes in β-Adrenergic Modulation on Rat Cardiac Excitation–Contraction Coupling

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Abstract

Previous studies have demonstrated that the ability of β-adrenergic receptor (βAR) stimulation to increase cardiac contractility declines with aging. In the present study, the control mechanisms of excitation–contraction (EC) coupling, including calcium current (I_{Ca}), cytosolic Ca^{2+} (Ca_i^{2+}) transient and contraction in response to βAR stimulation were investigated in ventricular myocytes isolated from rat hearts of a broad age range (2, 6–8, and 24 mo). While the baseline contractile performance and the Ca_i^{2+} transient did not differ markedly among cells from hearts of all age groups, the responses of the Ca_i^{2+} transient and contraction to β-adrenergic stimulation by norepinephrine (NE) diminished with aging: the threshold concentration and the ED_{50} increased in rank order with aging; the maximum responses of contraction and Ca_i^{2+} transient decreased with aging. Furthermore, the efficacy of βAR stimulation to increase I_{Ca} was significantly reduced with aging, and the diminished responses of the contraction and Ca_i^{2+} transient amplitudes to NE were proportional to the reductions in the I_{Ca} response. These findings suggest that the observed age-associated reduction in βAR modulation of the cardiac contraction is, in part at least, due to a deficit in modulation of Ca_i^{2+}, particularly the activity of L-type calcium channels. (*J. Clin. Invest.* 1994. 94:2051–2059.) Key words: aging • β-adrenergic receptor • calcium current • cytosolic calcium • contraction

Introduction

It has been widely documented that the efficacy of β-adrenergic receptor (βAR)[1] stimulation to modulate cardiovascular function declines with adult aging (see reference 1 for review). In humans, this decrease is manifested, in part, by an age-associated decrease in heart rate augmentation, left ventricular dilatation, and a diminished left ventricular ejection fraction during exercise (2). As plasma catecholamine levels increase with aging, particularly during stress (3), the age-associated decline in the efficacy of βAR system appears to be largely postsynaptic in origin. Accordingly, infusions of βAR agonists at rest elicit a lesser augmentation in heart rate or in left ventricular ejection fraction in older versus younger humans (4). In hearts and cardiac muscle isolated from rat heart, the direct contractile response to βAR stimulation has also been found to decline with aging (5–7). While more recent studies have indicated that this age-associated myocardial deficit is due, at least in part, to a failure of βAR agonists to increase the contractile strength of individual cardiac cells isolated from older versus younger hearts (8), it has not been established which particular control mechanisms of excitation–contraction (EC) coupling in cells of the older heart fail to respond adequately to β-adrenergic receptor stimulation.

In this study, the ability of βAR stimulation to enhance the cystolic free calcium (Ca_i^{2+}) transient and contraction elicited by excitation were measured simultaneously in single cardiac cells (9) isolated from rats of a broad age range (2–4, 6–8, and 24 mo). The effects of βAR stimulation to augment calcium current (I_{Ca}) of sarcolemmal L-type Ca^{2+} channels in cells from different aged hearts was also examined. Under control conditions, there was no systematic difference in the parameters of EC coupling, such as I_{Ca}, contraction and Ca_i^{2+} transient amplitudes, among cells from the three age groups. However, a generalized diminution in the responses of the Ca_i^{2+} transient and contraction amplitudes and of their kinetics to the physiological βAR agonist NE occurred with increasing age. The results indicate that a diminished contractile response to βAR stimulation with aging is due to a lesser increase in Ca_i^{2+} transient in older versus younger hearts, and that the relative failure of βAR stimulation to augment the Ca_i^{2+} transient amplitude and to reduce its duration in cells from older hearts can be explained by an age-associated failure of βAR stimulation to augment I_{Ca} and SR Ca^{2+} pump activity.

Methods

Preparation of isolated cardiac myocytes. Single ventricular cardiac myocytes were isolated from 2–4, 6–8 and 24-mo-old rat hearts by a standard enzymatic technique (8). Briefly, hearts of male Wistar rats from the Gerontology Research Center Colony were quickly removed under sodium pentobarbital anesthesia and retrogradely perfused with a low Ca^{2+}, collagenase bicarbonate buffer solution (36°C, pH 7.4). When the heart became soft, the perfusion was terminated, the left ventricle was mechanically dissociated and cells were suspended in Hepes buffer solution consisting of (mM): $CaCl_2$, 1.0; NaCl, 137; KCl, 5.0; dextrose, 15; $MgCl_2$, 1.3; NaH_2PO_4, 1.2; Hepes (5-*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid), 20; pH was adjusted to 7.4 with NaOH. Cells were stored at 37°C until ready for use.

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1. *Abbreviations used in this paper:* βAR, β-adrenergic receptor; Ca_i^{2+}, cystolic free calcium; dIFT/dt, the maximum rate of rise of Indo-1 fluorescence transient; EC, excitation-contraction; IFTA, Indo-1 fluorescence transient amplitude; $IFTt_{1/2}$, the time from stimulation to 50% relaxation of Indo-1 fluorescence transient; SR, sarcoplasmic reticulum; TA, cell twitch amplitude; VS, cell maximum shortening velocity.

Simultaneous measurements of cell length and Ca_i transient. Myocytes were loaded with the fluorescent Ca^{2+} probe, Indo-1 acetoxymethyl ester (Indo-1/AM) as previously described (9). 50 μg of Indo-1 AM (Molecular Probes, Inc., Eugene, OR) was added to 2.0 ml of cells in the Hepes-buffered solution (final Indo-1 AM concentration was 23 μM) for 8–10 min. The myocytes were then resuspended in Hepes-buffered solution and stored in the dark at room temperature for at least 1 h before use.

Following Indo-loading, cells were placed on the stage of a modified inverted microscope (model IM-35; Carl Zeiss, Inc., Thornwood, NY) equipped for simultaneous recording of Indo-1 fluorescence and cell length (9). Briefly, cells were perfused with Hepes-buffered solution containing 1 mM Ca^{2+} at room temperature (23°C) and were electrically stimulated by platinum field electrodes (2 ms square wave at two times threshold). The excitation wavelength was selected by a 350-nm interference filter (bandwidth 10 nm; Oriel Corp., Stratford, CT). Upon excitation at this wavelength, Ca^{2+}-bound and Ca^{2+}-free forms of Indo-1 emit light peaking at ~ 410 and 490 nm respectively (10). The ratio of emission intensity at 410 nm to that at 490 nm was computed offline and the resulting waveform used as an index of the Ca_i^{2+} transient. The peak amplitude of the transient is defined as the difference of the 410:490 ratio fluorescence recorded before electrical stimulation and peak fluorescence after electrical stimulation. The background autofluorescence and Indo-1 fluorescence signals were not changed by any of the drugs used in this study. Each cell was also simultaneously illuminated with red (650–750 nm) light through the normal brightfield path of the microscope. Cell length at rest and following excitation was monitored from the brightfield image of the cell by an optical edge tracking method using a photodiode array (Reticon model 1024; SAQ, Sunnyvale, CA) with a 5 ms time resolution (9).

Electrophysiological measurements. In other cells from hearts of three age groups, the L type Ca^{2+} channel current, I_{Ca}, was measured with voltage-clamp technique in the whole cell mode (11). The superfusion solution was the same as that used for cell length and Ca_i^{2+} transient measurements except that KCl was replaced by CsCl. Patch microelectrodes (3–5 MΩ) were filled with the solution containing (in mM): CsCl, 120; Hepes, 20; $MgCl_2$, 1.5; NaCl, 10; EGTA, 5; Mg^{2+}-ATP, 3; pH was adjusted to 7.2 with CsOH. All of the experiments were performed with a discontinuous switch clamp technique on an Axoclamp II amplifier (Axon Instrs., Inc., Foster City, CA) controlled by a specially designed hardware/software system used to simultaneously control the cell and acquire (at 2 kHz) the electrophysiological data.

In order to selectively examine L-type calcium channels in whole-cell current records, cells were voltage-clamped at −40 mV to inactivate the sodium current and T-type I_{Ca}. I_{Ca} was measured as the difference between peak inward current and the current at the end of the 200 ms pulse (12). Since in some cells I_{Ca} kinetics could not always be fit by a single exponential, the current inactivation rate was indexed as 63% of the decay time. Resting membrane potential was measured in the current clamp mode immediately following the breaking of the cell membrane. Cell membrane capacitance was measured as described previously (13). Briefly, to measure cell membrane capacitance (C_m), a small current pulse (ΔI) under current-clamp was applied and the time-course of membrane potential was recorded. Input resistance (R_i) was measured from the magnitude of the current pulse (ΔI) and the change in the steady-state membrane potential (ΔE_m), $R_i = \Delta E_m/\Delta I$. The membrane time constant (τ) was estimated from a single exponential fit of the time course of the change in E_m. The membrane capacitance was then estimated from $C_m = \tau/R_i$.

To measure the steady-state inactivation of I_{Ca}, the membrane potential was depolarized to +10 mV, a voltage that maximally activates the calcium channels, following a 2000-ms conditioning pulses to potentials between −50 and 0 mV. Currents at each test potential were normalized to the maximum current and plotted as a function of conditioning potential. The voltage dependence of I_{Ca} steady-state activation was calculated from the equation:

$$g = I/(E_m - E_{rev}) \quad (1)$$

where g is the membrane conductance, I is the peak current at a given potential E_m, and E_{rev} is the apparent reversal potential of I_{Ca} (+55 mV). The conductance at each test potential was normalized to peak conductance. The data were fit by a Boltzmann equation:

$$d_\infty = \{1 + \exp[-(V_m - V_{0.5})/k]\}^{-1} \quad (2)$$

$$f_\infty = \{1 + \exp[(V_m - V_{0.5})/k]\}^{-1} \quad (3)$$

where d_∞ is the steady-state activation; f_∞ is the steady-state inactivation; $V_{0.5}$ represents the half maximal activation or the half maximal inactivation point; k is the slope factor of the steady-state activation or inactivation curves.

Experimental protocol. Dose-response curves for NE (Sigma Chemical Co., St. Louis, MO) in cardiac cells of three age groups were implemented in an experimental protocol described in a recent study of this lab (8). Briefly, the contractile and Ca_i transient were measured in 5–15 cells as control values before addition of NE. After the superfusion solution was switched to the solution containing NE at certain concentration, the steady-state responses of contraction and Ca_i^{2+} transient to NE (after 10 min) were measured in 5–10 cells by random. For an individual heart, a complete dose-response curve including a control value and responses to six NE concentrations was obtained. Cells isolated from eight hearts of each age group were studied. In pilot experiments, in cells from young animals, a dose-response curve using each cell as its own control was highly comparable to the dose-response curve produced by the present method.

The effect of NE on I_{Ca} was measured in another series of cells from three age groups. Following the establishment of the current clamp, protocols for measuring the cell resting membrane potential and the cell membrane capacitance were executed. The patch clamp amplifier was then switched to the voltage clamp mode. In I_{Ca} experiments, each cell as its own control, i.e., I_{Ca} from a given individual myocyte was recorded before and 10 min after NE (10^{-7} M) exposure. In some experiments, Cd^{2+} (3×10^{-5} M) or nifedipine (10^{-6} M) were applied in the continued presence of NE.

Statistical methods. The effect of age on I_{Ca}, twitch contraction, and Ca_i^{2+} transient parameter responses to NE was tested via one way ANOVA, two factor ANOVA, repeated measures analysis of variance when appropriate (14). Post hoc analyses (Bonferonni) were performed to determine between which age groups significant age effects occurred.

Results

The baseline cell length and Indo-1 fluorescence transient (IFT) parameters before and after electrical excitation are presented in Table I. The cell length prior to excitation (diastolic cell length) increased with age, as noted previously (15). Neither the twitch amplitude (TA), i.e., extent of cell shortening relative to the resting length during the twitch contraction, nor the maximum shortening velocity (VS) varied with age. The time from stimulation artefact to 50% relaxation of twitch ($Tt_{1/2}$) tended to increase with age but this did not reach a statistical significance. Increases with aging were observed in the diastolic Indo-1 fluorescence ratio and in the Indo-1 fluorescence transient amplitude (IFTA) following excitation. The maximal rate of IFT increase (dIFT/dt) and the time from stimulation artefact to 50% relaxation of Indo-1 fluorescence transient ($IFTt_{1/2}$) did not significantly vary with aging.

Representative examples of the contraction and Indo-1 fluorescence transient before and after NE (10^{-6} M) are illustrated in Fig. 1. *A* shows that NE increases the amplitudes of the cytosolic calcium transient and contraction. The inset illustrates that VS and dIFT/dt are markedly accelerated by NE. The later suggests that βAR stimulation increases the rate and amount of sarcoplasmic reticulum (SR) Ca^{2+} release. In addition, the

Table I. Baseline Contraction and Indo-1 Fluorescence Transient Parameters in Single Rat Cardiac Myocytes of Varying Age

Age	Diastolic length	Twitch amplitude	Maximum twitch shortening velocity	Twitch $t_{1/2}$	Diastolic indo fluoresence ratio	Maximum rate of indo fluorescence ratio increase	Indo fluorescence ratio transient amplitude	Indo fluorescence ratio $t_{1/2}$
	μm	*% diastolic length*	*μm/s*	*ms*	*410/490 nm*	*ratio s^{-1}*		*ms*
2 mo (n = 104)	117.42±1.88*	6.30±0.32	62.69±3.70	551.71±12.48	0.952±0.020*	4.01±0.23	0.201±0.01‡	405.58±10.83
6–8 mo (n = 99)	130.02±1.50	6.70±0.31	74.40±4.67	579.62±13.98	1.123±0.028	4.60±0.30	0.228±0.012	393.68±8.19
24 mo (n = 71)	125.22±1.94	6.66±0.44	65.61±6.19	581.02±15.36	1.090±0.029	4.54±0.26	0.261±0.013	418.13±11.02
Overall age effect	0.001	NS	NS	NS	0.01	NS	0.002	NS

* $P < 0.05$, 2 mo vs 6–8 mo, and 24 mo; ‡ $P < 0.05$, 2 mo vs 24 mo.

diastolic cell length was decreased by NE. Both the relaxation times of the twitch and IFT were abbreviated by NE stimulation. To better illustrate the effects of NE on the kinetics of contraction and Ca_i^{2+} transient, the tracings of *A* are normalized to their peak amplitudes and plotted in *B*.

In additional experiments, the effect of NE and NE plus prazosin, an α_1-adrenergic blocker, on contraction amplitude was examined in both Indo-1–loaded and non-Indo-1–loaded cells. The increases in Ca_i^{2+} transient and contraction amplitude induced by NE (10^{-7} M) were not significantly affected by α_1-adrenergic receptor blocker, prazosin, at 1.0 μM (Fig. 2), but were completely abolished by βAR antagonist, propranolol, at 1.0 μM (data not shown).

The average concentration-response relations of NE for TA, VS, IFTA, and dIFT/dt from cells of all three age groups are presented in Fig. 3. With increasing age, the concentration-response curves for all parameters shift downward and rightward. The average concentration response relation for the NE-induced acceleration of the IFT and contraction relaxation rate are shown in Fig. 4. The NE-induced reductions of $Tt_{1/2}$ and $IFTt_{1/2}$ are also blunted with increasing age.

The statistical threshold concentration of NE, defined as the lowest concentration of NE required to produce a statistically significant change of the average value of a measured variable from its baseline value, increased in rank order with age for most contraction and Indo-1 fluorescence parameters. For example, the threshold concentrations of NE to increase contraction amplitude are 5×10^{-9} M, 10^{-8} M, and 5×10^{-8} M for cells from 2, 6–8, and 24 mo, respectively. In addition to an increase in the threshold concentration of NE, the concentration of NE required to elicit 50% of the maximal effect (ED_{50}, Table II) increased with age, indicating an age-associated decline in the sensitivity to NE. The maximum responses of twitch and IFT to NE exhibited a generalized, age-associated decrease (Table III). Prazosin did not alter the age-associated reductions in the contractile response to NE. On average, NE (10^{-7} M) in the absence and presence of prazosin (10^{-6} M) increased contraction amplitude of cells from 2-mo rat hearts to 234.84±17.68% of control and 237.93±24.83% of control, respectively, (n = 5). Similarly, in cells from 24-mo-old rat hearts, NE increased contraction amplitude to 169.08±20.23% of control and 162.62±16.35% of control (n = 5) in the absence and presence of prazosin (10^{-6} M), respectively.

During steady-state electrical stimulation, the magnitude and kinetics of Ca^{2+} release from the SR into the cytosol are reflected by the IFTA or dIFT/dt. The extent and the rate of

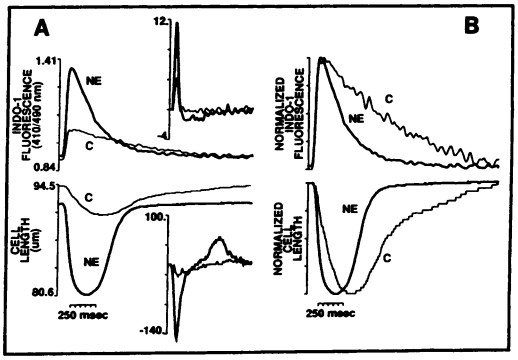

Figure 1. Effects of NE (10^{-6} M) on twitch and Ca_i^{2+} transient amplitudes and kinetics. (*A*) Tracings obtained in the presence and absence of NE in the same myocyte are superimposed. The Effects of NE on dIFT/dt and VS are shown in the inset. (*B*) The Ca_i^{2+} transient and twitch tracings of *A* are normalized to their peak amplitude to better illustrate the effect of NE on the acceleration of $t_{1/2}$ of both parameters.

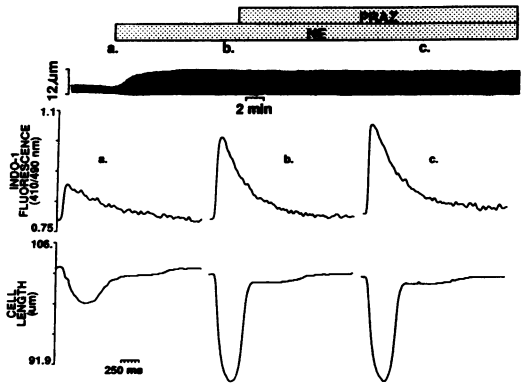

Figure 2. A representative example of the effect of NE (10^{-7} M) on contraction of a cell from 2-mo rat heart in the absence and presence of an α_1-adrenergic blocker, prazosin, at 10^{-6} M. (*Top panel*), continuous chart recording of cell contraction. (*Bottom panel*), simultaneous recorded Ca^{2+} transient and contraction tracings were obtained at the times indicated in the top tracing and show control (*a*), after NE (*b*) and after adding prazosin (*c*).

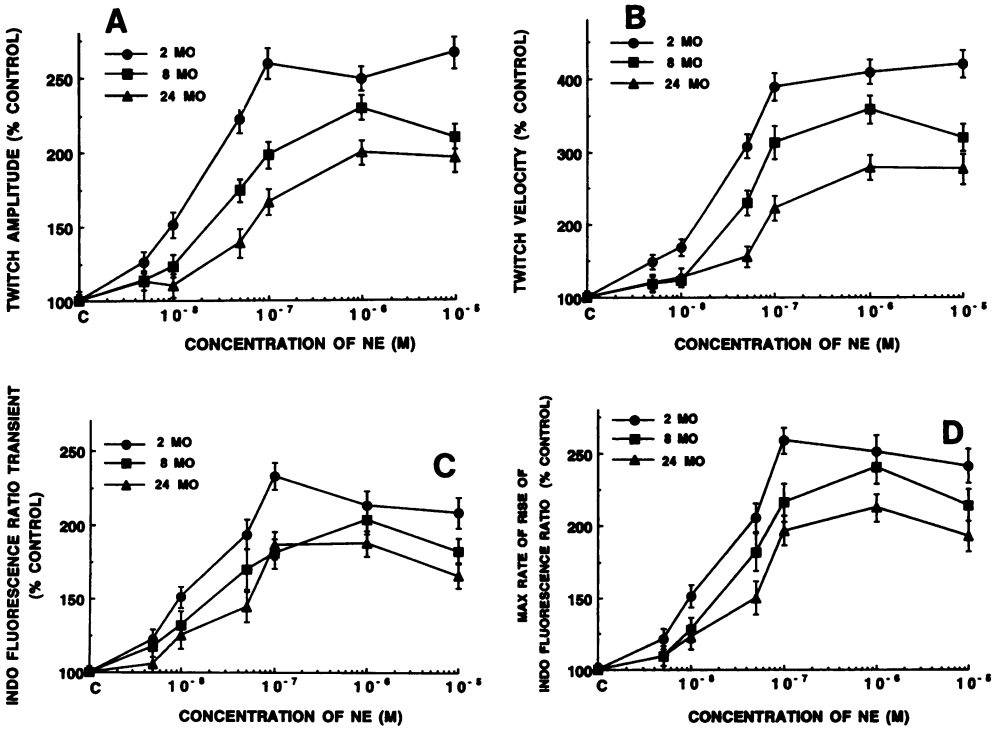

Figure 3. Average dose-response curves for responses of TA, VS, IFTA, and dIFT/dt to NE in cells of three age groups. Data are presented as percent of control values (listed in Table I). Each point represents the mean±SE of 35 to 50 cells. (*A*) For TA, the overall age effect is significant at $P < 0.001$ by analysis of two factors ANOVA. Significant differences (by Bonferonni) are: 2 vs 6 mo; 2 vs 24 mo; 6 vs 24 mo. (*B*) For VS, there is a significant ($P < 0.05$) interaction among the three age groups. (*C*) For IFTA, the overall age effect is significant at $P < 0.0001$ by analysis of two factors ANOVA. Significant differences (by Bonferonni) are: 2 vs 6 mo; 2 vs 24 mo. (*D*) For dIFT/dt, the overall age effect is significant at $P < 0.0001$, by analysis of two factor ANOVA. Significant differences (by Bonferonni) are: 2 vs 6 mo; 2 vs 24 mo. (Note that two factors ANOVA results are from concentration from 10^{-8} M to 10^{-6} M.)

myofilament shortening in response to the increase in Ca_i^{2+} after an excitation are reflected by TA and VS, respectively. The age-associated decreases in the response of IFTA and dIFT/dt to NE (Fig. 3, *C* and *D*) suggest that the NE induced increase in Ca^{2+} released from the SR into the cytosol decreases with age, and that this accounts for the observed age-associated reduction in TA and VS (Fig. 3, *A* and *B*). This point was further investigated by examining the relationship between the NE-induced changes in Ca_i^{2+} transient (either IFTA or dIFT/dt) and the NE-induced changes in contraction (either TA or VS). IFTA vs TA or dIFT/dt vs VS are comparable in all age groups, suggesting that age-associated alterations in myofilament sensitivity to cytosolic Ca^{2+} do not occur (Fig. 5, *A* and *B*).

β-adrenergic stimulation augments Ca^{2+} influx via L-type sarcolemmal Ca^{2+} channels (16, 17), which triggers SR Ca^{2+} release in cardiac myocytes (18). Furthermore, the SR Ca^{2+} release is tightly controlled by the I_{Ca} in a graded manner (19, 20). Thus, the relative decrease in the ability of NE to augment IFTA or dIFT/dt with aging (Fig. 3 and Tables II and III) may result from a relative reduction in the ability of NE to augment L-type Ca^{2+} channel current in cells from older hearts. Therefore, we studied the I_{Ca} response to NE at 10^{-7} M, i.e., that concentration producing the approximate maximum responses in Figs. 3 and 4, in cells of three age groups. Table IV lists the average membrane resting potential, cell membrane capacitance, peak I_{Ca} at a test potential of 0 mV, and the I_{Ca} normalized by the cell membrane capacitance for cells from hearts of the three age groups. The significant age-associated increase in membrane capacitance is in agreement with hypertrophy of myocytes from older hearts, as shown by the increase of cell dimensions (15) (Table I). The peak I_{Ca} was also significantly increased with aging. However, I_{Ca} normalized for cell membrane capacitance, i.e., the I_{Ca} density did not vary with age as shown previously (21). Representative examples of the effect

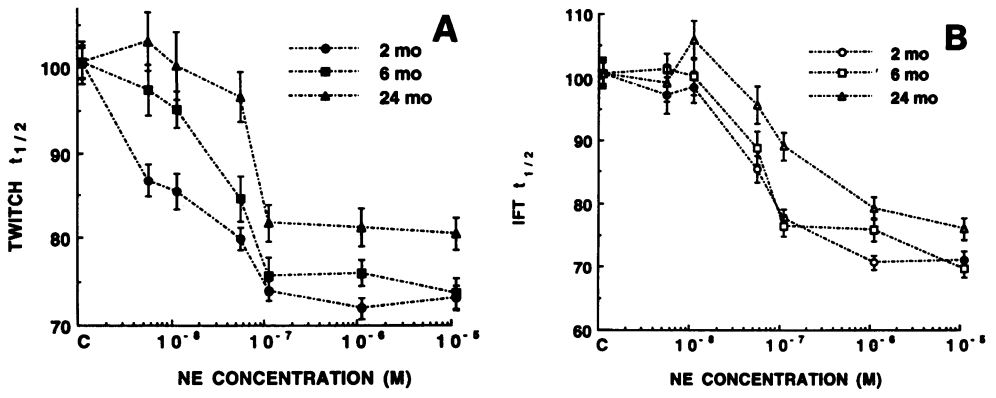

Figure 4. Average effect of age on the responses of twitch duration, $Tt_{1/2}$ (time from stimulus artefact to 50% cell relengthening), and Indo-1 transient duration, $IFTt_{1/2}$ (time from stimulus artefact to 50% reduction of the Indo-1 transient), to NE. (*A*) For $Tt_{1/2}$, the overall age effect is significant at $P < 0.0001$ by two factors ANOVA. Significant differences (by Bonferonni) are: 2 vs 6 mo; 2 vs 24 mo; 6 vs 24 mo. (*B*) For $IFTt_{1/2}$, the overall age effect is significant by two factors ANOVA $P < 0.0001$. Significant differences (by Bonferonni) are: 2 vs 24 mo; 6 vs 24 mo.

Table II. Concentration of NE Required to Produce 50% of Maximum Effect on Contraction and Indo Fluorescence Transient Parameters

	TA	VS	IFTA	dIFT/dt
	$\times 10^{-8}$ M	$\times 10^{-8}$ M	$\times 10^{-8}$ M	$\times 10^{-8}$ M
2 mo				
(n = 8)	1.76±0.38	2.70±0.41	1.30±0.35	1.79±0.24
6–8 mo				
(n = 8)	3.66±0.50	4.27±0.61	2.11±0.18	2.75±0.31
24 mo				
(n = 8)	5.77±0.64*	8.15±0.62*	4.08±0.86‡	4.67±0.65*
Age effect	0.0001	0.0001	0.0072	0.0007

TA, twitch amplitude; VS, maximum twitch shortening velocity; IFTA, Indo-1 fluorescence transient, an index of the cytosolic Ca^{2+} transient; dIFT/dt, maximum rate of rise of indo-1 fluorescence transient.
* $P < 0.05$ 24 mo vs 2 mo, and 6–8 mo; ‡ $P < 0.05$ 24 mo vs 2 mo.

Table III. Average Maximal Effect of NE on Contraction and Indo Fluorescence Transient Parameters (Percentage of Control)

	TA	VS	IFTA	dIFT/dt
2 mo				
(n = 55)	266.7±10.5*	420.7±18.6‡	233.1±9.1‡	259.2±9.1‡
6–8 mo				
(n = 42)	230.1±8.4	359.1±19.7§	204.1±10.6	241.2±12.1
24 mo				
(n = 54)	199.9±8.2	278.8±17.2	187.9±8.7	212.7±9.3
Age effect	0.0001	0.0001	0.002	0.003

TA, twitch amplitude (percentage of diastolic cell length); VS, maximum twitch shortening velocity; IFTA, indo-1 fluorescence transient; dIFT/dt, maximal rate of rise of indo-1 fluorescence transient.
* $P < 0.05$ 2 mo vs 6–8 mo and 24 mo; ‡ $P < 0.05$ 2 mo vs 24 mo; § $P < 0.05$ 6–8 mo vs 24 mo.

of NE on I_{Ca} of cells from 2-mo hearts are illustrated in Fig. 6, *A* and *B*. The peak I_{Ca} increased by about twofold and the inactivation rate of I_{Ca} was slightly accelerated. The blockade of the recorded currents by Cd^{2+} and nifedipine, L-type calcium channel blockers, are shown in *A* and *B*, respectively, indicating that the recorded current is L-type I_{Ca}. Fig. 6 *C* shows the time course and the stability of the effect of NE to augment the I_{Ca} amplitude of the cell in *A*. Fig. 6 *D* shows the peak I_{Ca} current-voltage relation in another cell, measured before and 10 min after the addition of NE to the bathing solution. The effect of the Cd^{2+} blockade on the current-voltage relation is also shown.

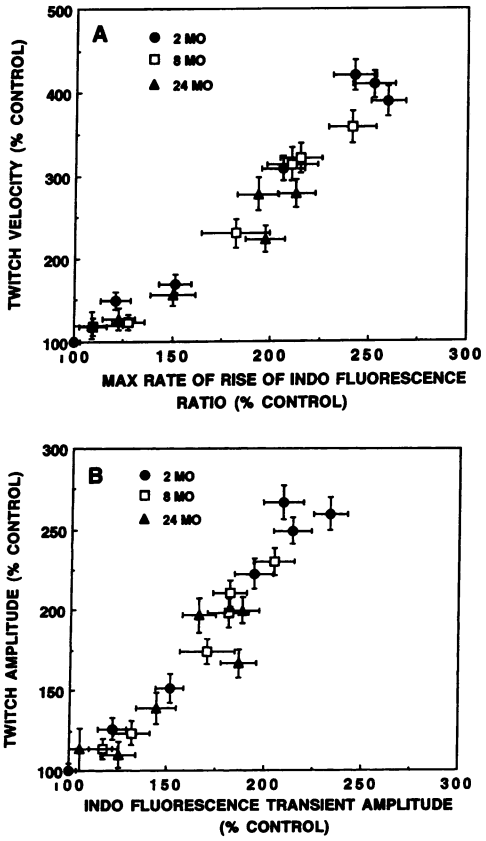

Figure 5. Relationship between the NE induced change in the maximal rate of rise of Indo-1 transient (dIFT/dt) or Indo-1 transient amplitude (IFTA) and twitch contraction velocity (VS) or twitch amplitude (TA) for cells of three age groups. (*A*) Increase in VS was plotted against the increase in dIFT/dt (data from Fig. 3, *B* and *D*) for all three age groups. (*B*) Increase in TA was plotted as a function of the increase in IFTA in the presence of NE for three age groups (data from Fig. 3, *A* and *C*).

Under control conditions, the calcium current density (normalized I_{Ca})-voltage relations of three age groups are superimposable (Fig. 7 *A*). In contrast, the extent of augmentation of I_{Ca} by NE decreases with increasing age (Fig. 7 *A*). In addition, the test potential giving rise maximal I_{Ca} was shifted about 6 mV towards negative potentials in all three age groups during NE exposure. When the increase in I_{Ca} is presented as percent of control values, and plotted as a function of test potential (Fig. 7 *B*), it is clearly demonstrated that the effects of NE are strongly dependent on the test voltage. The stimulatory effect of NE was greater at negative test potentials and decreased at more positive test potentials in all age groups. Importantly, the age-associated reduction in I_{Ca} response to NE is greater in the negative than in the positive test potential range.

The voltage dependencies of the steady-state activation and inactivation of the L-type calcium channel were estimated in cells from hearts of three age groups (Fig. 8). In the absence of NE stimulation, no age differences were observed either in

Table IV. Membrane Potential, Membrane Capacitance, Peak I_{ca}, and I_{ca} Normalized to Cell Membrane Capacitance

Age	n	RP (mV)	Cm (pF)	I_{Ca} (nA)	I_{Ca}/Cm (pA/pF)
2 mo	18	−64.71±1.16†	124.3±12.8*	0.72±0.07*	5.33±0.49
8 mo	18	−67.60±2.26	186.5±14.6	1.09±0.06	5.38±0.30
24 mo	18	−71.77±1.08	221.8±18.0	1.27±0.09	5.34±0.60
Age effect		.012	0.0002	0.0001	NS

RP, resting membrane potential; Cm, membrane capacitance; I_{Ca}, peak amplitude of calcium current elicited by depolarizing the cell at 0 mV from holding potential of −40 mV; I_{Ca}/Cm, peak I_{Ca} normalized to membrane capacitance. * $P < 0.05$ 2 mo vs 6–8 mo and 24 mo. † $P < 0.05$ 2 mo vs 24 mo.

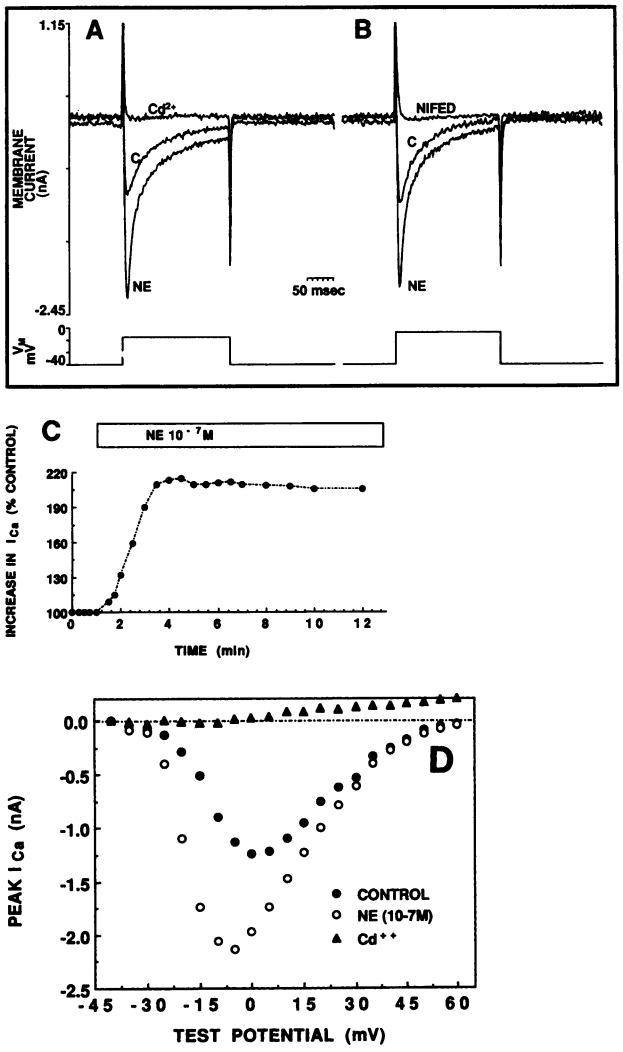

Figure 6. Representative effect of NE (10^{-7} M) on the amplitude of calcium current (I_{Ca}), on the current-voltage relationship of the I_{Ca} and on the time course of the increase in I_{Ca} in 2-mo-old rat ventricular myocytes. (*A* and *B*) Currents were elicited by a depolarizing test pulses from a holding potential of −40–0 mV for 200 ms at 0.5 Hz. Current tracings recorded under control conditions, in the presence of NE and in the presence of Cd^{+2} at 3×10^{-5} M or nifedipine (10^{-6} M) plus NE are superimposed. (*C*) Time course of the effect of NE on I_{Ca} in the cell shown in *A*. (*D*) Current-voltage relations of I_{Ca} for the same cell as in *A* and *C*. The cell was depolarized from a holding potential of −40 mV to test potentials from −35 to +60 in 5 mV increments. Pulse duration was 200 ms and stimulation rate was 0.5 Hz.

the half maximal activation or inactivation points ($V_{0.5}$) or in the slope factors (k) of the activation and inactivation curves. These results are in agreement with a recent report (21). In contrast, in the presence of NE, the inactivation curves of cells from 2–4-mo and 6–8-mo hearts were significantly shifted toward negative potentials as compared with that of cells from 24-mo hearts that did not exhibit this shift ($V_{0.5}$ are −26.82±1.55 mV, −28.94±1.68 mV and −22.36±1.50 mV for 2, 6–8 and 24 mo, respectively. $P < 0.01$ 24 mo vs 2 and 6–8 mo, $n = 18$ for all age groups). The slopes of the inactivation curves in the presence of NE were also less steep than that of cells from 24 mo (k values are 4.62±0.20, 4.66±0.20, and 5.50±0.25 for 2, 6–8, and 24 mo, respectively. $P < 0.01$ 24 mo vs 2 and 6–8 mo, $n = 18$ for all age groups). In contrast, there are no age-associated differences in $V_{0.5}$ and k of the steady-state activation curves from cells of three age groups although all three curves were shifted to the negative direction by about 6 mV after application of NE.

It is generally accepted that the majority of the increase in Ca_i^{2+} that activates contraction originates from the SR Ca^{2+} release via the "calcium-induced calcium release" mechanism (18, 19). To examine whether the ability of the SR to amplify the triggering I_{Ca} is altered with aging, the relationship between changes in I_{Ca} and the corresponding changes in TA and IFTA was examined in cells of three age groups. Fig. 9 illustrates the relationships between the NE (10^{-7} M)-induced increases in peak I_{Ca} and the increase in IFTA or TA in the three age groups. The data points lie about a line through the origin, suggesting that the age-associated decreases in the IFTA and TA are accompanied by a similar extent reduction of I_{Ca}. This result suggests that the observed age-associated reductions in the NE induced increases in I_{Ca} could, at least in part, be responsible for the age-associated reductions in the increases of Ca_i^{2+} transient (IFTA) and contraction amplitude (TA) induced by NE.

Discussion

In the present study, a generalized age-associated deficit in the modulation of EC coupling by βAR stimulation induced by NE

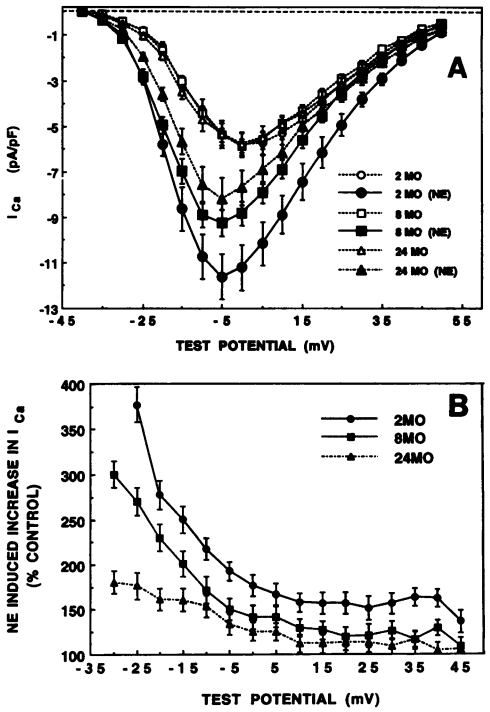

Figure 7. The average effect of 10^{-7} M NE in cells of three age groups on I_{Ca} normalized to cell membrane capacitance. The experimental protocol is the same as that used in Fig. 6 *D*. (*A*) Current-voltage relation for cells of three age groups before and during exposure to NE. In the absence of NE, no significant age differences in I_{Ca} density are present at any voltage. (*B*) The relative increase in I_{Ca} during exposure to NE is plotted as a function of test potentials. The overall age effect on the response of I_{Ca} to NE is significant (from test potentials −25 to +45 mV) at $P < 0.0001$ by repeated measures analysis of variance. Significant differences are: 2 vs 6 mo; 6 vs 24 mo; 2 vs 24 mo. Each data point represents mean and standard error ($n = 18$).

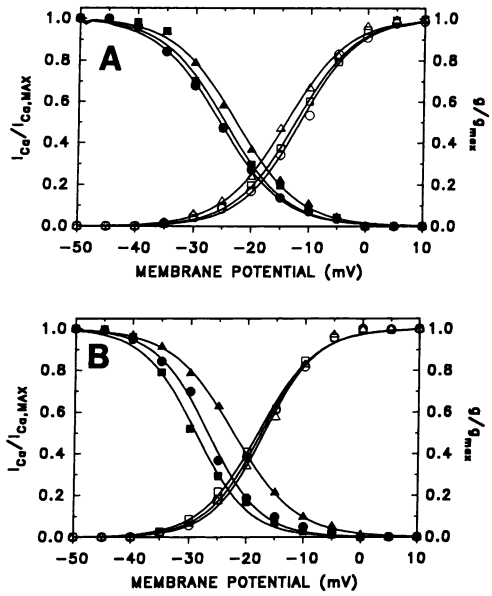

Figure 8. Voltage dependence of I_{Ca} steady-state activation (SSA, *open symbols*) and steady-state inactivation (SSI, *solid symbols*) of cells from three age groups. SSA and SSI were estimated by protocols described in Methods. The curves were fitted to a Boltzmann equation to compare half maximal activation and inactivation potential points ($V_{0.5}$) and slope factors (k) among age groups. (*A* and *B*) The voltage dependence of SSA and SSI of cells of three age groups before and after exposure to NE 10^{-7} M, respectively (n = 18 for each point). Circles represent 2 mo; squares represent 6–8 mo; and triangles represent 24 mo.

in cardiac cells has been demonstrated by measuring I_{Ca}, Ca_i^{2+} transient and contraction in cells from hearts of a broad age range. Both the sensitivity and the maximum responses of cardiac myocytes to βAR stimulation decline with aging (Tables II and III) and the concentration of NE to cause threshold effects increases with aging. Before NE, most of parameters of EC coupling did not differ from each other among the three age groups (Tables I and IV and Fig. 7), suggesting that the declining ability of cells to adequately respond to the stress of βAR stimulation is not due to marked changes in intrinsic EC coupling mechanisms.

The specific major findings of the present study are: (*a*) the increase in twitch amplitude and twitch velocity of rat ventricular myocytes in response to β-adrenergic receptor stimulation induced by NE is markedly decreased with aging (Fig. 3, *A* and *B*, Tables II and III). A similar effect of age to reduce the magnitude of the NE response was observed previously in non-Indo-1 cells (7). (*b*) Age-associated reductions occurred in the responses to NE of the Indo-1 fluorescence transient amplitude and its maximal rate of rise (Fig. 3, *C* and *D*, Tables II and III), indices of excitation induced Ca^{2+} release from the SR. (*c*) The kinetics of the relaxation of the Ca_i^{2+} transient and contraction are accelerated to a lesser extent by NE in cells from older than in cells from younger hearts (Fig. 4). This finding suggests that the Ca^{2+}-sequestering activity of SR responses to βAR stimulation decreases with age, probably due to an age-related decreased phosphorylation in SR phospholamban by βAR stimulation (6). (*d*) While the average I_{Ca} density under control conditions does not vary with age, the average increase of I_{Ca} current induced by NE significantly declines with age (Fig. 7). The extent of the age-associated diminution of response of I_{Ca}

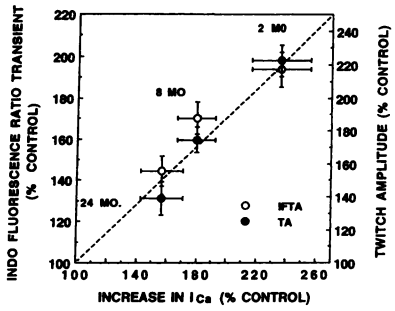

Figure 9. Relationship of the relative NE (10^{-7} M)-induced increases in I_{Ca} and twitch amplitude or Indo-1 fluorescence transient amplitude for three age groups. Data are presented as percentage of control values (Table I and IV).

to βAR stimulation is similar to that of Ca_i^{2+} transient and contraction (Fig. 9).

In this study NE was used as a βAR agonist because it is an important physiological neurotransmitter in the mammalian heart. Although NE is a mixed α- and β-adrenergic receptor agonist, the selective α_1-adrenergic receptor antagonist, prazosin, at 1.0 μM affected neither contractile response to NE in cells from 2 mo nor that in cells from 24 mo (Fig. 2). This result indicates that the NE-induced positive inotropic effect in rat cardiac cells is mediated almost exclusively via activation of β-adrenergic receptors. This observation is supported by our previous studies (8, 12, 22). In contrast, in single, isolated rat cardiac cells used in this and previous studies, phenylephrine does evoke an α-adrenergic receptor response, but the effects of phenylephrine are quite different from those of βAR activation by NE. For example, the positive α-adrenoceptor inotropic effect induced by phenylephrine is associated with only a slight increase in Ca_i transient, suggesting that the myofilament sensitivity to Ca^{2+} is increased by α-adrenoceptor stimulation (23, 24); and, the contraction duration in rat and rabbit cardiac myocytes increases rather than decreases (23, 24).

EC coupling in heart cells is initiated as the sarcolemmal calcium current, I_{Ca}, becomes activated by the membrane depolarization. The transient influx of Ca^{2+} through the sarcolemmal calcium channels both initiates and grades the contractile response of heart cells via control of the release of Ca^{2+} from SR (19, 20). In addition to supplying a "trigger" for calcium induced calcium release from SR, the sarcolemmal Ca^{2+} influx is important for the replenishment of SR Ca^{2+} by the SR Ca^{2+}-ATPase pump (25), and it also directly contributes about 5–10% of the Ca_i^{2+} transient during an electrical stimulated contraction (26). Therefore, I_{Ca} may be the key element responsible for a decreased response of cytosolic calcium and contraction to βAR stimulation with aging. Under control conditions, neither the I_{Ca} density nor the current density-voltage relationship is altered with aging, although peak I_{Ca} was larger in cells from older hearts than that of cells of younger hearts (Table IV and Fig. 7). These results are in agreement with a recent study on the single rat ventricular myocytes from the same rodent strain (21). More importantly, these results demonstrate that the ability of NE to augment peak I_{Ca} declines with aging, suggesting that the ability of NE to increase the availability of Ca^{2+} channels (17), or to promote calcium channel switching from a short to a long opening state (27), becomes reduced with aging.

The effect of βAR stimulation to enhance peak I_{Ca} in the present study, or in other studies of cardiac cells (28), is markedly voltage dependent. Fig. 7 *B* clearly demonstrated that the effect of NE is greater at more negative test potentials than at positive ones for all age groups. Furthermore, this study also

clearly shows that the age effect to reduce I_{Ca} response to βAR stimulation was also greater at more negative test potentials and decreased as the test potential increased. Previous studies have suggested that at more depolarized membrane potentials L-type calcium channels proteins become a less favorable substrate for cAMP-dependent phosphorylation or a better substrate for phosphoprotein phosphatase (29, 30). If these hypotheses regarding the voltage-dependent phosphorylation are correct, a voltage-dependent reduced effect of the βAR stimulation in older hearts could indicate an age-related reduction in the extent of calcium channel phosphorylation by protein kinase A.

In this study we observed that the kinetics of I_{Ca}, as indicated by the voltage dependence of the steady-state activation and steady-state inactivation, do not differ with age under control conditions. This finding is consistent with the findings of a previous study (21). However, the $V_{0.5}$ of the I_{Ca} steady-state inactivation of 2 and 6–8-mo cells were significantly shifted toward the negative direction whereas that of the 24-mo cells did not shift after NE. This might also indicate that the response of calcium channel gating to NE is altered with aging. Since there was no age-associated difference in the steady-state activation of I_{Ca} after NE, the window current (the overlap of the steady-state activation and inactivation curves, Fig. 8 *B*) was greater in cells of 24-mo hearts than that of 2 and 6–8-mo hearts in the presence of NE. The physiological importance of L-type window current has not been fully understood. In ventricular cells, the window current may delay termination of the action potential plateau, and possibly contribute to contractile activation and refilling of the SR. Under pathophysiological conditions, the window current at plateau potentials may contribute to the genesis of arrhythmic early afterdepolarizations (31, 32). This might be a possible contributor to the increased tendency for arrhythmias during βAR stimulation in older than in younger hearts (5).

These results suggest that with aging the reduced ability of βAR stimulation to induce an increase in I_{Ca}, the trigger for SR Ca^{2+} release, is likely the major reason for the lesser augmentation of calcium-induced calcium release from SR by βAR stimulation in cells from older versus younger hearts (Fig. 9). The relative reduction in the augmentation of I_{Ca} by βAR stimulation with aging may also lead to an age-associated reduction of the βAR stimulation induced increase in the SR Ca^{2+} loading in cells from older hearts. A lesser decrease in the $IFTt_{1/2}$ by NE with aging suggests that βAR stimulation induced acceleration of Ca^{2+} reaccumulation by the SR, thought to be related to cAMP-dependent phosphorylation of SR phospholamban, decreases with age. Age differences in SR Ca^{2+} release in response to βAR stimulation may also relate, in part, to age-associated changes in SR Ca^{2+} release channel properties following NE, which could not be measured in this study.

Studies in suspensions of single rat ventricular cells have demonstrated that the NE-induced increase in phosphorylation of troponin I (TnI), which is thought to decrease the Ca^{2+} binding to troponin C and to reduce the increase in contraction amplitude for a given $[Ca_i^{2+}]$, decreases with age (6, 33). These results, however, indicate that the relationship between the NE-induced changes in IFTA and TA (Fig. 5 *A*) or the relationship between the NE-induced changes in dIFT/dt and VS (Fig. 5 *B*) do not markedly differ among age groups. Prior study in skinned fibers indicates that in the absence of βAR stimulation the steady state force-Ca^{2+} relationship does not vary with age (34). Thus, decreased TnI phosphorylation may not directly relate to the myofilament sensitivity to Ca_i^{2+}, or the effect is offset by additional factors not measured in this study.

It is concluded from the results of this study that the age-associated diminution of the I_{Ca} increase and of the acceleration of SR Ca^{2+} cycling in response to NE are largely responsible for the diminished augmentation of Ca_i transient by NE, which, in turn, results in an age-associated decrease in cardiac myocyte contractile response to βAR stimulation. In addition, an age-associated diminution in the NE-induced augmentation of the SR Ca^{2+} loading or a difference in SR Ca^{2+} release channel properties may also contribute to the age-associated changes in the Ca_i^{2+} transient and contraction following βAR stimulation. However, the later issues require further study.

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