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THE AGING HEART: CHANGES IN THE PHARMACODYNAMIC ELECTROPHYSIOLOGICAL RESPONSE TO VERAPAMIL IN AGED RABBIT HEARTS

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The aim of this study was to investigate whether the L-type calcium current ($I_{Ca.L}$) may be altered in aged hearts and whether the classical calcium antagonist verapamil may exhibit altered pharmacological profile in aged hearts. We examined male New Zealand rabbits aged either 6 months or 26 months. To examine $I_{Ca.L}$ whole-cell patch-clamp technique was performed on isolated cells. Moreover, activation-recovery intervals (ARI) of isolated hearts (Langendorff method) were assessed using an epicardial 256 channel mapping system. We found that the $I_{Ca.L}$ density, normalised to the cell volume was significantly reduced ($p<0.001$). Maximum conductance was also significantly decreased ($p=0.01$) and steady state inactivation was shifted to more positive potentials in aged hearts ($p<0.001$). A slightly reduced effect of β-adrenergic modulation of the $I_{Ca.L}$ in aged hearts, and a significantly reduced effect of carbachol on isoprenaline-stimulated $I_{Ca.L}$ in aged hearts was observed. L-type α1c subunit, SERCA2-ATPase and the Na^+/Ca^{2+}-exchanger expression were neither significantly different in atrial and ventricular tissues nor between young and old animals. Using the mapping system, isolated hearts were exposed to verapamil (0.005, 0.01, 0.02, 0.05 μM/L). While verapamil did not affect ARI in young hearts, in aged hearts ARI was concentration-dependently reduced and the negative inotropic effect of verapamil was significantly attenuated in aged hearts ($p<0.05$). From these results we conclude that there are distinct alterations in the electrophysiology of $I_{Ca.L}$ (reduced maximum conductance, a shift of the steady state inactivation) in the aged heart which may influence the response to verapamil.

Key words: *calcium, L-type calcium current, aging, verapamil, carbachol, isoprenaline, cardiac electrophysiology*

INTRODUCTION

The percentage of elderly people in western industrialised countries is steadily increasing. Currently, 31 million people are older than 65 years in the US, accounting for 12% of the total population. By 2025 a total of 20% of the population will be older than 65. A significant proportion of this population receives drug treatment because of underlying cardiovascular diseases. Within this context it is well known that the anatomy and histology of the heart changes with age characterised by increased deposition of collagen and fat (1) which influences the biophysical properties in the aged heart (2). On the cellular level a progressive prolongation of the action potential has been reported (3). These changes have been explained by a decreasing density of the transient outward potassium current, I_{to}, (4, 5) and a progressive prolongation of the decay time of the L-type calcium current, $I_{Ca,L}$, during aging. In addition to the prolongation of the action potential-duration (APD), the sensitivity to β_1-adrenergic stimulation is reduced progressively with aging (reviews: (6, 7)). However, only little is known, whether these morphological and electrophysiological changes will alter the response to pharmacological agents. It is well known that the pharmacokinetics of a drug will be altered with age, since the metabolic and renal clearance will change, but there is only little information on the question whether the pharmacodynamics may also change with the age-related alteration of cardiac histology and electrophysiology.

Thus, it was the aim of the present study to investigate whether the electrophysiology of $I_{Ca,L}$ is altered in aged hearts and whether the widely used antiarrhythmic and antihypertensive drug verapamil (calcium antagonist) may exhibit an altered pharmacological profile in aged hearts. In order to exclude pharmacokinetic phenomena like decreased renal clearance we decided to investigate the drug effects in isolated hearts from rabbits of different age (6 months (=young sexually mature adults) *versus* 26 months (=aged)). This model

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has been established previously (2). As only few electrophysiological data on single ventricular cells from aged rabbit heart are available (reviews: (6, 7)) patch clamp experiments on $I_{Ca,L}$ were performed to characterise this current under our experimental conditions. Finally, we evaluated whether the expression of the most prominent calcium handling proteins such as L-type channel subunit α1c, SERCA2a, Na^+/Ca^{2+} exchanger, is changed in atrial and ventricular tissues obtained from young and aged rabbits.

MATERIALS AND METHODS

Two groups of male white New Zealand rabbits have been investigated: young sexually mature adult rabbits (6 months body weight: 2430±65 g; heart weight: 8.0±0.3g, n=14) and old rabbits (26±2 months body weight: 4400±180g; heart weight: 21.2±2.4g, n=14) (normally fed *ad libitum*, Rollie, Langenhagen, FRG). All experiments were performed in accordance with the ethical rules of the Guide for the Care and Use of Laboratory Animals (US National Institute of Health) and the German laws for animal welfare, granted by the local ethical authorities.

Mapping experiments in isolated hearts

The method of heart preparation as well as the epicardial potential mapping has been described in detail previously (8): briefly, male white New Zealand rabbits were treated with 1000 IU/kg heparin i.v. 5 min before they were stunned by a sharp blow on the neck and killed rapidly by subsequent exsanguination. The heart was excised, prepared and perfused according to the Langendorff-technique at constant pressure of 70 cm H_2O with Tyrode's solution of the following composition (in mM/L): Na^+ 161.02, K^+ 5.36, Ca^{2+} 1.8, Mg^{2+} 1.05, Cl^- 147.86, HCO_3^- 23.8, PO_4^{2-} 0.42 and glucose 11.1, equilibrated with 95% O_2 and 5% CO_2 (pH=7.4). The surface temperature of the heart amounted to 37°C. The hearts were connected to a 256 channel mapping system HAL3 (ELSA, Aachen, Germany, temporal resolution: 4 kHz/channel; amplitude resolution: 0.04 mV, interchannel coupling <-60 dB; bandwidth of the system: 0.5 Hz - 20 kHz, data were not filtered) as described previously (9). 256 AgCl electrodes were cast in 4 polyester plates (in 8*8 orthogonal matrices with 1 mm interelectrodes distance), which were attached to the surface of the hearts in an elastic manner, so that they could easily follow the movements of the hearts without dislocation. Electrode plates were located at the front wall (64 electrodes), at the left wall (64 electrodes), at the right wall (64 electrodes) and at the back wall (64 electrodes). The hearts were paced with rectangular pulses of double threshold (3Hz) at the right atrium near the vena cava superior at a rate of 3 Hz.

We administered verapamil (young *vs*. old) in cumulative concentrations of 0.005, 0.01, 0.02 and 0.05 µM/L, each concentration being applied for a period of 10 minutes. The concentration range covers the common free plasma concentrations. Each experimental series was carried out with n=7 experiments.

Epicardial potential mapping was performed in each experimental phase during periods of constant cycle length of at least 4 min, in order to make it possible to compare the activation patterns (of single heart beats) or their alterations. In addition, the functional parameter maximum developed systolic left ventricular pressure (LVP) and coronary flow (CF) was assessed continuously as described (8). The activation time points at each electrode were determined as the time point of the minimum of the derivative of the voltage, $t(dU/dt_{min})$ (8, 10), and the repolarisation time points during the T-wave as the time point of the maximum of the derivative of the voltage, $t(dU/dt_{max,})$ (8, 11). After automatic determination the activation and repolarisation time points were verified (or corrected if necessary) manually by the experimentator. From these data for each electrode an activation-recovery-interval (ARI, corresponding to the epicardial potential duration) was calculated. The corresponding local distribution of ARI was analysed for each area of the heart (*i.e*. front, left, right or back wall) calculating the standard deviation of ARI at 64 electrodes and expressed as ARI-dispersion. From the activation time points an activation sequence was determined. We determined those electrodes which were activated before any of the neighbouring ones and defined them as "breakthrough-points" which can be considered as the origins of epicardial activation (12).

In order to allow a quantitative and comparative description of the activation process for each electrode an activation vector was calculated. This vector is based on the difference in activation times between neighbouring electrodes, *i.e*. if a given electrode #A activates earlier than its neighbour #B, the velocity of the conductance between both can be calculated as well as the direction of conduction. For one electrode up to 8 of these vectors were calculated and summed up vectorially as described earlier (13) giving a resulting vector of activation propagation for the given electrode #A. These vectors describe the direction and the apparent velocity of local activation.

In a subsequent step, we used these vectors and their directions for comparison of heart beats under control conditions with heart beats under drug influence. Comparing different heart beats, the percentage of similar vectors (VEC) between heart beats in the various experimental phases compared with those under control conditions was determined. Each vector under drug influence (or at any experimental phase) was compared with its corresponding vector of a heart beat under control conditions and vectors deviating not more than 5° from their original direction were considered to be similar. Then the percentage of similar vectors was calculated. The critical value beneath which arrhythmia often occur (see above) for VEC-similarity is 10% as determined in previous studies (8, 9, 14, 15) Thus, VEC characterises the geometry of the epicardial activation process, and represents the beat similarity of the cardiac impulse as compared to heart beats under control conditions. Decreasing values of VEC indicate the progressive deviation from the initial (control) activation pattern. In addition, the duration of the QRS-complexes in the epicardial electrograms was analysed.

Experiments on isolated cells

Isolation of ventricular myocytes

Ventricular myocytes were isolated enzymatically by a procedure adapted from (16). The animals were sacrificed as described above. The thorax was rapidly opened and 10 ml heparin were injected into vena cava inferior. The heart was removed and retrogradely perfused using the following solutions (for composition see *Table 1*): (1) Ca^{2+}-free Tyrode solution, 5 min); (2) high K^+ solution (HPS, 5 min); (3) HPS with trypsin (0.625 mg/ml, 6-8 min or 10-11 min in case of young or old animals, respectively); (4) HPS with collagenase (type L, 1 mg/ml; 14-15 min in case of young, 17-18 min in case of old animals). Isolated cells were stored up to 8-10 h in oxygenated standard Tyrode's solution at room temperature until use.

Cell morphology

Cell volume of isolated ventricular myocytes was estimated from microscopic bright field images. In bright field images only the length and the width of a cell can be measured, the third

dimension for each cell was estimated by shifting the plane of focus. This estimation showed cell height to be in proportion with the cell width, *i.e.* relatively wide cells were higher than those with a small width. However, as the estimation of the height is relatively inaccurate we decided to set the height to 10 µm in all cases of cells from the young animals. In the case of the old animals we assumed that the myocytes had grown in height by the same amount as in width, which was supported by light microscopical estimations of the cell height. In aged myocytes the width was found to be increased by a factor of 1.57 compared to the young ones, thus the height was set to be 15.7 µm in aged myocytes. The expansion of T-tubules was disregarded for the calculation of the cell volume. The number of nuclei per cell was determined following staining with 4',6-diamidino-2-phenylindole (DAPI). After isolation, myocytes were washed once in PBS and stained with DAPI (Serva, Heidelberg, Germany; 0.1 µg/ml in 100% methanol) for 15 min at 37°C. Thereafter, cells were washed several times in PBS and investigated on a fluorescent microscope (Zeiss IM 405, Zeiss, Oberkochen, Germany; excitation wavelength: 340 nm, emission: 488nm).

Electrophysiological recordings

Standard whole-cell patch-clamp recordings (17) were carried out at 36±1C using the solutions given in *Table 1*. Patch pipettes of 2-4MΩ were pulled from borosilicate glass (Hilgenberg, Malsfeld, Germany). A single-electrode continuous-voltage clamp amplifier (L/M EPC 7; List Medical Electronic, Darmstadt, FRG or Axopatch 200-A, Axon Instruments Foster City, CA, USA) was used. Cell capacitance, series resistance (4-8MΩ), and junction potentials were compensated using the circuitry of the respective amplifier. Voltage-clamp protocols, data acquisition, and data storage were accomplished using pClamp 6.0 (Axon Instruments). Membrane potential and current recordings were sampled at 5.5-50 kHz using a 12-bit A/D converter (Digidata 1200, Axon Instruments).

Action potentials (APs) were triggered by current injection *via* the patch electrode (0.5-1.0 nA, 4-6 ms; 1 Hz) in K^+-based external and internal solutions (*Table 1*). AP duration was evaluated from the time of decay from 10 to 90% of repolarisation (APD_{10-90}) using averaged APs of 10 consecutive stimulations.

$I_{Ca,L}$ was elicited by rectangular voltage pulses after block of overlapping current systems in Cs^+-based solutions (*Table 1*). Gapped double-pulse protocols composed of two test-clamps (first: 400 ms; consecutively increasing in 5 mV steps from –40 to 60 mV and second: 400 ms; 10 mV constant) separated by a 2 ms gap (-40 mV) were used in order to determine current/voltage relationship, steady-state activation (d_∞), and steady-state inactivation (f_∞). Cells were stimulated at 0.2 Hz. Recovery of L-type channels from inactivation was investigated by double pulses (400 ms, 10 mV each) separated by a gap (-40 mV) of stepwise increasing duration (2-1200 ms). Stimulation frequency was 0.1 Hz.

Cell capacitance was calculated routinely at every cell prior to capacitance compensation from the capacitive current elicited by a fast ramp clamp decreasing from -40 to -50 mV (slope: 5.5 mV/ms). For evaluation all current amplitudes were normalised to cell capacitance. $I_{Ca,L}$ peak current/voltage relationships were fitted as published elsewhere (18) For averaging data from different cells, membrane currents were normalised to the cell capacitance.

Effects of isoprenaline and carbachol on isolated cardiomyocytes

In order to investigate the modulation of the $I_{Ca,L}$ by autonomic transmitters cells were held in the voltage-clamp mode at -50 mV, and trains of depolarising pulses lasting 20 ms were applied to a test potential of 0 mV at a frequency of 0.2 Hz.

Table 1. Composition of solutions used for myocyte isolation and patch-clamp recordings (in mM/L).

	myocyte isolation			external solutions		internal solutions	
	standard Tyrode	Ca^{2+}-free Tyrode	High K^+ solution (HPS)	K^+-based	Cs^+-based	K^+-based	Cs^+-based
NaCl	140	140	4	140	---	5	5
KCl	5.4	5.4	10	5.4	---	130	---
K-glutamate	---	---	130	---	---	---	---
CsCl	---	---	---	---	5.4	---	100
Choline-Cl	---	---	---	---	140	---	---
$MgCl_2$	1	1	1	1	1	---	---
$CaCl_2$	1.8	---	0.025	1.8	---	---	---
$BaCl_2$	---	---	---	---	2	---	---
4-AP	---	---	---	---	2.5	---	---
TEA-Cl	---	---	---	---	---	---	30
EGTA	---	2.6	---	---	---	10	10
Hepes	5	5	4	5	5	5	5
Mg-ATP	---	---	---	---	---	2.5	2.5
glucose	11	11	11	11	11	---	---
BSA [g/l]	1	---	---	---	---	---	---
trypsin-inhibitor [g/l]	167	---	---	---	---	---	---
pH	7.4 (NaOH)	7.4 (NaOH)	7.4 (KOH)	7.4 (NaOH)	7.4 (CsOH)	7.4 (KCl)	7.4 (CsOH)

Current-voltage (I/V) relationships were determined by applying 150 ms lasting depolarising voltage steps from test potentials of -40 mV to 40 mV in 10 mV steps. Membrane capacity was determined on line using the ISO 2 acquisition software program (MFK, Frankfurt, FRG). Data were acquired at a sampling rate of 10 kHz, filtered at 1 kHz, stored on hard disk and analysed off-line using the ISO2 analysis software package (MFK). Substances were applied only after establishment of stable $I_{Ca,L}$. We tested the effects of 100 nM/L isoprenaline, followed by additional 1 mM/L carbachol or of 1 mM/L carbachol alone. The linear regression between control amplitude of peak $I_{Ca,L}$ prior to carbachol (CCh) application and after wash-out of CCh was used as an estimate for $I_{Ca,L}$ run-down. For calculation of the CCh-dependent $I_{Ca,L}$ depression the wash-out value was defined as the peak current taken on the linear regression in the same time-point as the maximal inhibition of $I_{Ca,L}$ by CCh. The glass cover slips containing the cells were placed into a temperature-controlled (36±1°C) recording chamber and perfused continuously with extracellular solution by gravity at a rate of 1 ml/min. Substances were applied by exchanging the solution in the chamber, a 90% volume exchange was achieved within approximately 20 sec. The composition of the solutions used is given in *Table 1*.

Biochemical studies

Western blot analysis

Total cell lysates (40 µg/lane) of each sample (n=3) were subjected to SDS-PAGE on 7.5% gels. Protein was transferred electrophoretically to a nitrocellulose membrane. Equal transfer among lanes was verified by reversible staining with Ponceau red. Immunoblotting was performed with antibodies directed against the L-type channel α1c subunit (rabbit polyclonal, 1:1000; Alomone Labs, Jerusalem, Israel), SERCA2a ATPase (mouse monoclonal, 1:500, Calbiochem, Bad Soden, Germany), Na^+/Ca^{2+} exchanger (rabbit polyclonal, 1:2000, Dianova, Hamburg, Germany) and troponin T (mouse monoclonal, 1:1000, Calbiochem) as described before (19). Detection was performed with the enhanced chemi-luminescence technique (ECL, Amersham, Braunschweig, Germany). Densitometrical analysis of immunoblots was performed with the gel-documentation system (Gel-Doc) by Biorad using the analysis software Multianalyst (Bio-Rad, Munchen, Germany). All data consist of a minimum of at least three independent experiments from three different animals. Data were normalised to troponin T expression from the respective blot, detected by re-exposing the stripped blot to an anti troponin-T antibody. Data are presented relative to expression in atria from young individuals.

Chemicals

All chemicals used in this study were of analytical grade. All chemicals were purchased from Sigma (Deisenhofen, Germany), except heparin and trypsin which were from Serva (Heidelberg, Germany) and Boehringer (Mannheim, Germany), respectively.

Statistics

If not stated otherwise all values are given as MEANS±SEM of n experiments in each series. Significance was analysed using analysis of variance for comparison of multiple groups. If ANOVA indicated significant differences either Wilcoxon rank test for paired observations or Mann-U-test for unpaired observations (mapping data) or Student t-test after Bonferroni correction (isolated cardiomyocytes, data are given as MEANS±SD) were performed. For the biochemical experiments statistical comparisons were made by Student t-test, two sided with adjustment for multiple comparisons. For all investigations the level of significance was $p<0.05$.

RESULTS

Age dependent differences in cell morphology

Cell morphological investigations revealed a marked hypertrophy of myocytes derived from hearts of old rabbits. The cell volume increased by a factor of 3.26 from 23.6±6.8 pl (n=94) in young rabbits to 76.9±20.4 pl (n=65) in the old animals. Likewise the mean electrical cell capacitance, that is directly correlated to membrane surface, is markedly increased from 108.0±36.8 pF (n=73) to 260.7±97.5 pF (n=66). For both

Table 2. Characterization of L-type Ca^{2+} current in young and old rabbits.

	young			old			*p*
	mean	SD	n	mean	SD	n	
maximum current density [pA/pF]	-10.18	3.16	29	-8.71	2.87	30	0.07
maximum conductance [pS/pF]	254.4	44.5	29	217.1	61.9	30	0.01
reversal potential [mV]	54.6	3.7	29	55.6	3.9	30	0.28
steady-state activation:							
$V_{½}$ [mV]	-1.1	3.2	29	-2.7	4.8	30	0.14
K	5.6	3.6	29	4.6	2.5	30	0.22
steady-state inactivation:							
$V_{½}$ [mV]	-14.2	2.4	29	-10.8	3.3	30	<0.001
K	4.6	1.1	29	4.1	1	30	0.07
time course of inactivation (clamp potential: 10 mV):							
τ_1 [ms]	3.01	1.98	24	3.82	3	18	0.3
τ_2 [ms]	24.3	6.1	24	27.2	5.5	18	0.12
recovery from inactivation:							
τ [ms]	82.1	25.8	17	100.8	31.5	24	0.051
stimulation by isoprenaline (100 nM) %	178	111	5	85	71	5	0.16
depression by CCh (1 mM) %	70	32	5	41	12	5	0.01

parameters the differences proved to be highly significant ($p<0.001$). In contrast, the total number of nuclei per cell did not change during aging. In cells of young and old animals mean values of 1.92±0.44 (n=87) and 1.92±0.51 nuclei per cell (n=65) were found.

Electrophysiological investigations on isolated hearts

We found that the epicardial ARIs were not affected by verapamil in young rabbits as could be expected. However, in hearts from aged rabbits the ARIs were dose-dependently significantly shortened, the difference between young and aged being also significant (*Fig. 1A*). We found a small, but significant, prolongation of ARI in aged versus young hearts at baseline (control ARI (ms) values after 60 min equilibration: young: 138±4; aged: 150±2.7; aged *vs*. young: p=0.028).

Verapamil is well known to cause negative inotropic effects. Thus, verapamil led to a marked and significant decrease in left ventricular pressure (LVP) in hearts from young rabbits: while without verapamil LVP was not different between young and aged hearts (young: 105±5 mmHg; aged: 90±6 mmHg; n.s.), verapamil led to a concentration-dependent significant decline in LVP in young hearts by 55±2% at the highest concentration ($p<0.01$). This negative inotropic effect - although still present- was significantly attenuated in hearts from aged animals (*Fig. 1B*): in aged hearts LVP was reduced by only 27±2% ($p<0.05$). The difference between young and aged hearts was significant ($p<0.01$) for the whole concentration-response curve.

Coronary flow under control conditions was 29±2ml/min in young and 49.5±5 ml/min in aged hearts ($p<0.01$ aged *vs*. young). However, taking the different heart weight (young: 8.5±0.5 g, n=7) and aged rabbits (20.1±3.5 g, n=7) into account, the coronary flow/heart weight ratio was not significantly different and was 3.2±0.4 ml/min*g (young) or 2.6±0.5 ml/min*g (aged) (n.s.). In young hearts, coronary flow slightly decreased (30.7±1.9 (0.005 µM/L), 25.2±1.5 (0.01 µM/L),

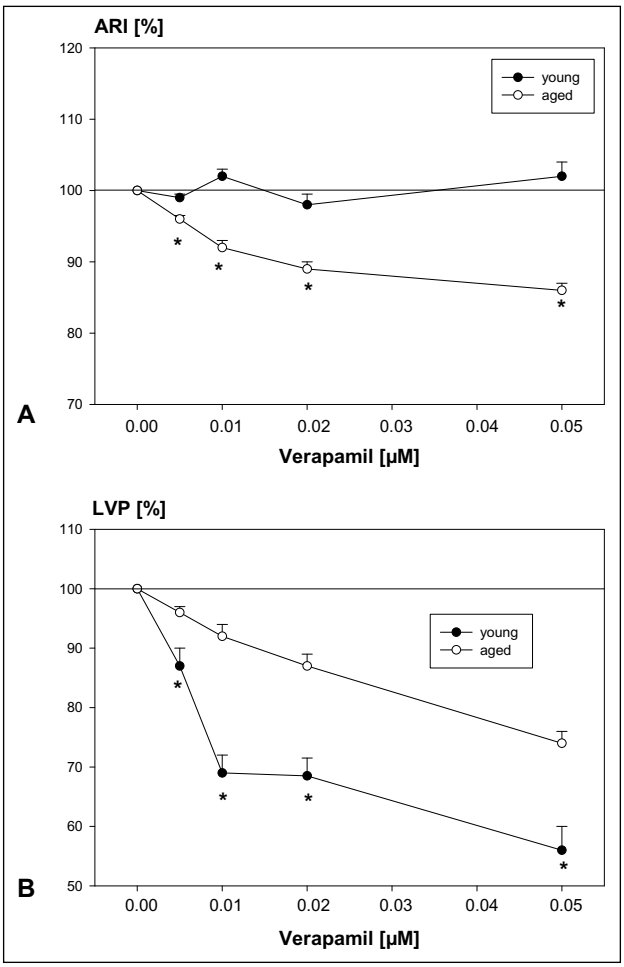

Fig. 1. (A) Percentual changes in epicardial activation-recovery interval (ARI) at 256 sites of the isolated rabbit heart under the influence of verapamil. Significant differences between young (n=7) and aged (n=7) rabbits are marked by an asterisk. (Control values (=100%): ARI [ms] after 60 min equilibration: young: 138±4; aged: 150±2.7; p=0.028). (B) Percentual changes in developed left ventricular pressure (LVP) of the isolated rabbit heart under the influence of verapamil. Significant differences between young (n=7) and aged (n=7) rabbits are marked by an asterisk. (Control values: LVP [mm Hg] after 60 min equilibration: verapamil series: young: 105±5; aged: 90±6; n.s.).

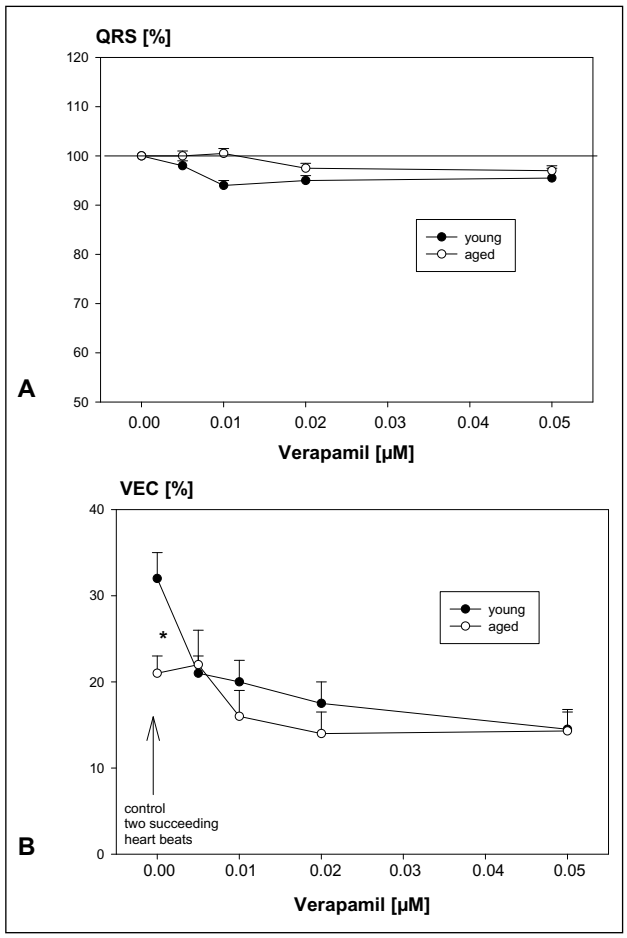

Fig. 2. (A) Percentual changes in duration of the QRS-complex of the epicardial potentials of the isolated rabbit heart under the influence of verapamil. Significant differences between young (n=7) and aged (n=7) rabbits are marked by an asterisk. (Control values: QRS [ms] after 60 min equilibration: verapamil series: young: 21±0.9; aged: 30±0.7; $p<0.05$). (B) Changes of the percentage of similar vectors (VEC) as compared to vectors under baseline conditions. For comparison, the maximum similarity of two successive heart beats is indicated. Changes are given for rabbit hearts under the influence of verapamil. Significant differences between young (n=7) and aged (n=7) rabbits are marked by an asterisk.

27.1±1.8 (0.02 µM/L), 23.2±2.5 (0.05 µM/L)) with verapamil. In aged hearts, however, coronary flow remained on a nearly constant level under verapamil (54.6±1.1 (0.005 µM/L), 56.2±1.8 (0.01 µM/L), 55.6±1.2 (0.02 µM/L), 54.2±2.2 (0.05 µM/L)). Taking the negative inotropy into account, the coronary flow/LVP ratio was enhanced under the influence of verapamil by +65±15% (young) or by +55±18% (aged) (maximum effect at 0.05 µM/L; aged *vs*. young: n.s.).

The duration of the ventricular QRS-complex was generally prolonged in aged versus young hearts (control values: QRS (ms) after 60 min equilibration: young: 21±0.9; aged: 30±0.7). Under the influence of verapamil the QRS-duration remained constant in both groups of hearts (*Fig. 2A*). There was no significant influence of verapamil on ST. Regarding the activation pattern, we observed that under control conditions in young hearts 32±2% of the vectors of two heart beats 30 minutes apart were similar, whereas in aged hearts the similarity was significantly lower and reached only 21±2% ($p<0.05$). Infusion of verapamil led to a concentration dependent decrease in the percentage of similar vectors to a similar extent in young (14.5±1% at 0.05 µM/L) and aged hearts (14±1% at 0.05 µM/L) (*Fig. 2B*).

Electrophysiological investigations on isolated cells

Action potential recordings

Since mapping experiments detected a significantly prolonged ARI in aged rabbit, we measured exemplary action potentials from isolated myocytes, and found slightly prolonged $APD_{10\text{-}90}$ (232.9±46.5 ms, n=5, young) *vs*. 277.6±68.8 ms, n=8, aged). The number of cells was however too low for statistical analysis.

L-type Ca^{2+} current

The verapamil sensitive L-type Ca^{2+} current ($I_{Ca,L}$) was characterised by means of standard rectangular double-pulse

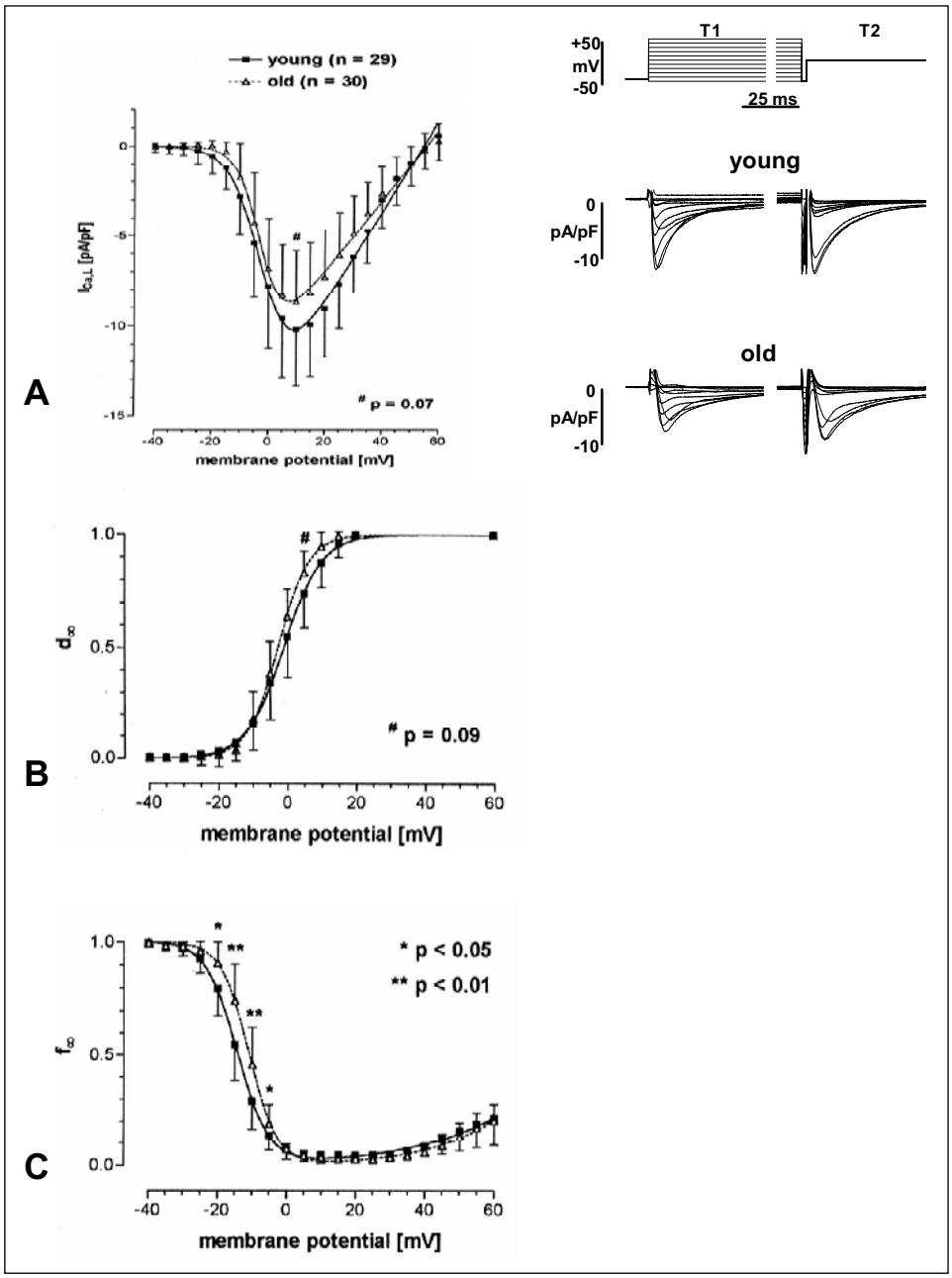

Fig. 3. (A) Characterisation of L-type Ca^{2+} current ($I_{Ca,L}$) in myocytes of young (squares; n=29) and old (triangles); (n=30) rabbits. Current/voltage relationship is normalized to cell capacitance. On the right side of the panel original current tracings of young and old rabbits and the pulse protocol are shown. (B) Steady-state activation (d_∞). Mean values were fitted by a Boltzmann function. (C) Steady-state inactivation (f_∞). Data were fitted by the sum of two Boltzmann functions.

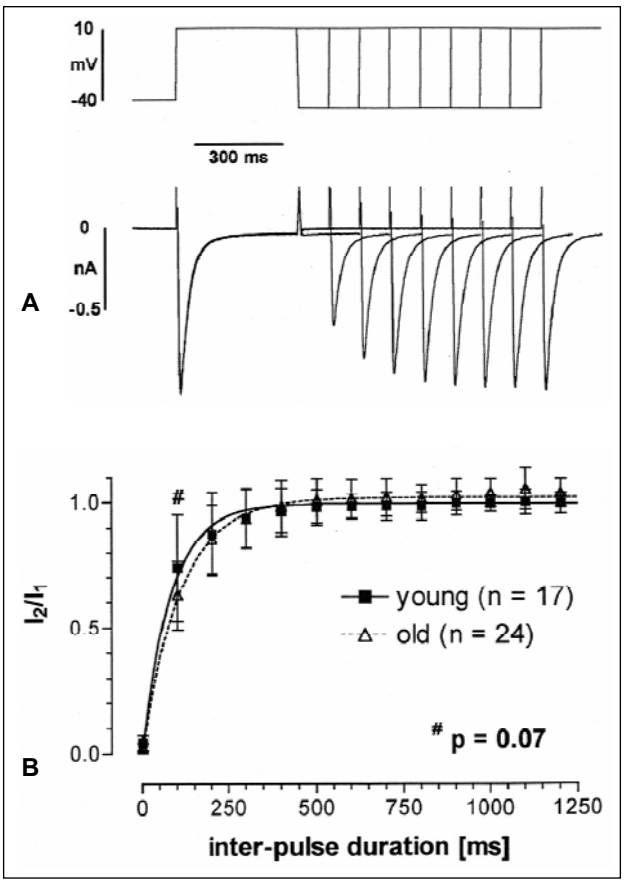

Fig. 4. Recovery of L-type Ca^{2+} current from inactivation. (A) Voltage protocol used for determination of recovery (upper trace) and corresponding original current recording (lower trace). (B) Relative recovery from inactivation calculated as the quotient of peak current amplitude during the second (I_2) and first (I_1) test pulse. Mean data obtained in myocytes of young (squares; n=17) and old (triangles); n=24) rabbits are plotted as function of the inter-pulse duration. Data were fitted by monoexponential functions.

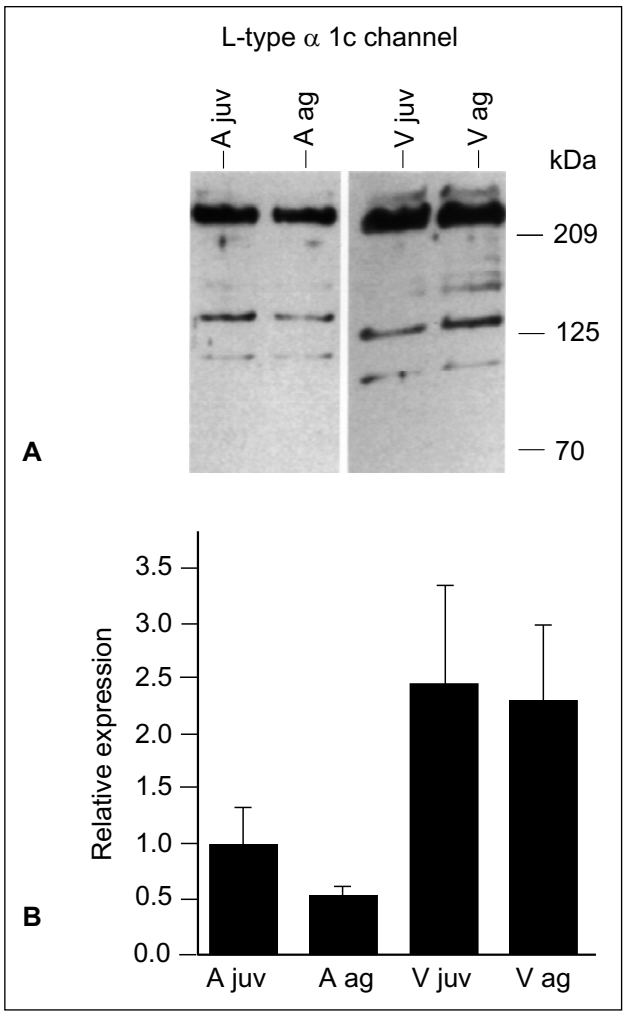

Fig. 5. Cardiac expression of the L-type α1c channel subunit in left atria and left ventricle of young and adult rabbits. Cellular lysates were subjected to SDS-PAGE (40 µg protein/lane), immunoblotted with anti L-type α1c antibody primary antibody and visualised by a chemiluminescence technique. Protein expression levels in left atria (A) and ventricles (V) form young (juv) and aged (ag) animals are shown as detected by immunoblotting (panel A). Bar graph (panel B) presents mean relative expression normalised to atria from young animals as measured by densitometry. Data are normalised to troponin-expression measured by re-exposure of immunoblots to an anti troponin-t antibody (n=3).

protocols. From these recordings conventional peak current-voltage characteristics, steady-state activation (d_∞) and inactivation (f_∞) and the time course of inactivation were derived (*Fig. 3, 4*).

In myocytes isolated from both age groups the $I_{Ca,L}$ showed the well-known time course of a rapid activation followed by an exponential inactivation (not shown). To quantify the time course of inactivation the original current traces were fitted by the sum of two exponential functions. The fast time constant τ_1 did not change during aging whereas the slow time constant τ_2, was slightly although not significantly, increased in myocytes derived from aged rabbits (*Table 2*). The current-voltage relationships were U-shaped in both age groups with maximum inward currents at 10 mV (*Fig. 3A*). Current densities were slightly lower in cells derived from old hearts. Maximum conductance was significantly reduced in myocytes of aged animals (217.1±61.9 pS/pF aged cardiomyocytes *vs*. 254.4±44.5 pS/pF young cardiomyocytes; p=0.01). The maximum inward current amplitude normalized to cell capacitance decreased from -10.2 pA/pF to -8.7 pA/pF during aging (p=0.07). The reversal potential, in contrast, was found to be unchanged during aging (*Table 2*).

As cells become larger during lifetime cell surface grows in the second power while the volume grows in the third power, provided that volume increment is equal in all directions and membrane folding remains unchanged. An unchanged current density will then lead to a decrease in the peak calcium influx per volume unit during hypertrophy. On the basis of the values recorded in this study the $I_{Ca,L}$/volume ratio decreased significantly (p<0.001) during aging from 46.6±1.38pA/pl to 29.52±2.44 pA/pl, *i.e*. by 36.6%.

The steady-state activation curves obtained for either age group were very similar (*Fig. 3B*). The potential of half-maximum activation and the slope factor k did not differ significantly (*Table 2*).

Steady-state inactivation curves changed significantly during aging (*Fig. 4C*, *Table 2*). This became obvious in the negative potential range: the potential of half-maximum inactivation was shifted by +3.4 mV in myocytes of old animals. The slope factor k was not changed. At positive membrane

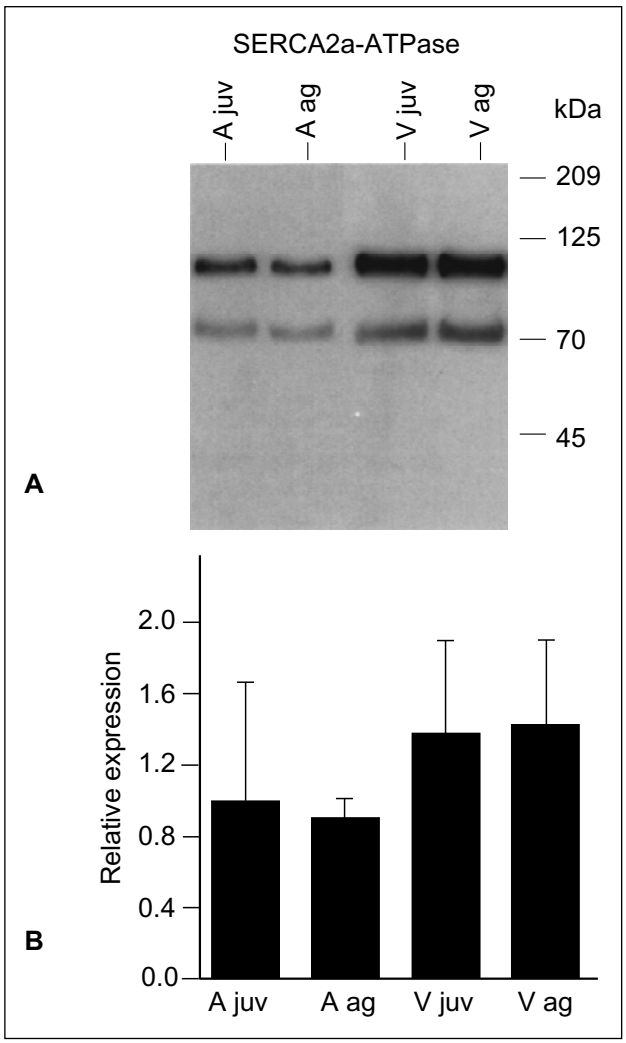

Fig. 6. Cardiac expression of the SERCA2a-ATPase in left atria and left ventricle of young and adult rabbits. Cellular lysates were treated as described in *Fig. 6*. Protein expression levels in left atria (A) and ventricles (V) form young (juv) and aged (ag) animals are shown as detected by immunoblotting (panel A). Bar graph (panel B) presents mean relative expression as measured by densitometry. Data are normalised as described in *Fig. 5*.

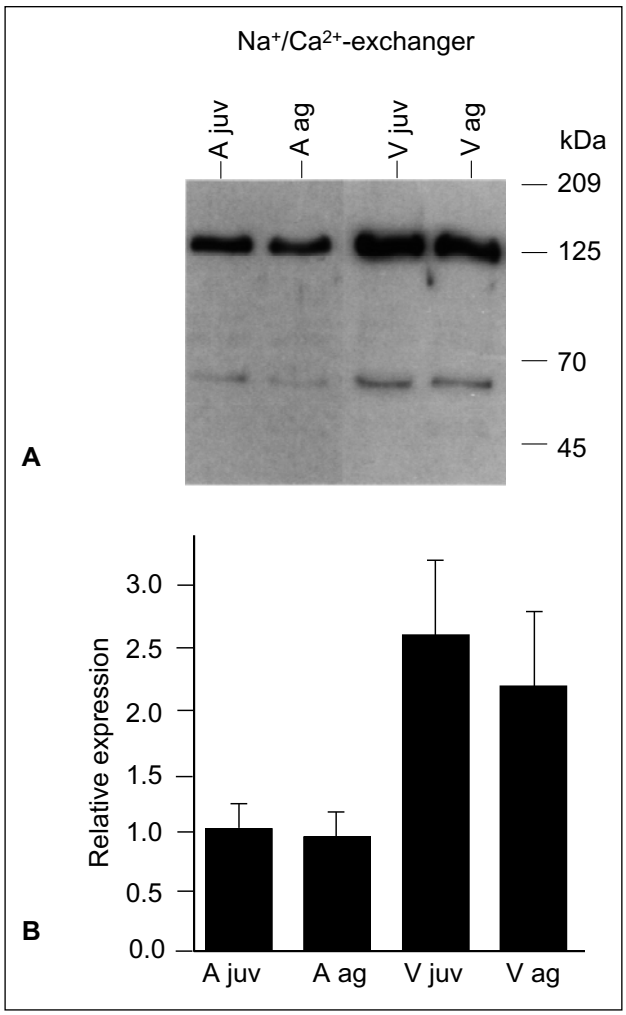

Fig. 7. Cardiac expression of the Na^+/Ca^{2+} exchanger in left atria and left ventricle of young and adult rabbits. Cellular lysates were treated as described in *Fig. 6*. Protein expression levels in left atria (A) and ventricles (V) form young (juv) and aged (ag) animals are shown as detected by immunoblotting (panel A). Bar graph (panel B) presents mean relative expression as measured by densitometry. Data were calculated and are normalised as described in *Fig. 5*.

potentials both inactivation curves are congruent showing a decrease of steady-state inactivation for membrane potentials >10 mV. Recovery from inactivation was assessed by means of double-pulse protocols with increasing inter-pulse gap (*Fig. 4A*). The relative amount of recovery was calculated by dividing the peak current amplitude elicited during the second pulse by the peak current amplitude elicited during the first pulse. In both, cells from young and aged animals full recovery was achieved within less than 500 ms. The time courses could be fitted by monoexponential functions that showed a small deviation for the short times (<300 ms) and superimposed almost completely later on (*Fig. 4B*). The time constant characterising the aged cardiomyocytes is slightly prolonged (p=0.051; cf. *Table 2*).

Effects of isoprenaline and carbachol on isolated cardiomyocytes

The modulation of $I_{Ca,L}$ by the autonomous nervous system plays an important role in cardiac function. In ventricular cardiomyocytes it is crucially important in the regulation of inotropy. Thus, we tested, whether any quantitative differences between young and aged cardiomyocytes could be detected in the β-adrenoceptor mediated- and the muscarinic regulation of $I_{Ca,L}$. Similarly as observed in guinea pig (20) and frog cardiomyocytes (21), basal $I_{Ca,L}$ was not depressed by the muscarinic receptor agonist carbachol (CCh, 1 mM/L) in the two groups of rabbit cardiac myocytes. In both, young and aged cardiomyocytes isoprenaline (100 nM/L) stimulated strongly $I_{Ca,L}$ density by 178±111% (±SD; n=5) and 85±71%(±SD; n=5) (p=0.16). Subsequent application of 1 mM/L CCh resulted in a pronounced depression of $I_{Ca,L}$ density by 70±32% (n=5) and 41±12% (n=5) (p=0.01).

Biochemical findings

Total cellular lysates obtained from rabbit atria and ventricle of young and old animals were subjected to SDS-Page gel electrophoresis and specific antibodies against L-type Ca^{2+}

channel $\alpha 1_c$ subunit, SERCA2a ATPase and Na^+/Ca^{2+} exchanger were used. To control for equal loading and protein content all immunoblots were stripped and re-exposed to an antibody against troponin-T (which was unchanged with age). We found that the expression of L-type $\alpha 1_c$ protein seemed lower in atrial samples than in ventricular tissue. However, there was no significance in the expression pattern between samples obtained from young and aged hearts (*Fig. 5A*,*B*). Regarding SERCA2a ATPase expression there was no significant difference between samples obtained from atrial and ventricular tissue neither from the same age group nor between the age groups (*Fig. 6A*,*B*). Finally, we examined the expression of the Na^+/Ca^{2+} exchanger protein in samples from rabbit atria and ventricles. We found that the expression of this protein was not altered in aged animals as compared to young, neither in atrial nor ventricular tissue (*Fig. 7A*,*B*).

DISCUSSION

The results obtained from the mapping study in Langendorff hearts indicate a significant difference in the response of hearts from young and aged rabbits to verapamil. The lower effect of verapamil on left ventricular pressure in aged hearts could be a hint on altered channel density, altered calcium handling, altered properties of the cardiac calcium channels, an altered binding of verapamil to its binding site, or a different transduction from drug binding into channel inhibition (or mechanical tissue alterations). The fact that verapamil shortened ARI in aged but not in young hearts could mean an altered calcium handling or that $I_{Ca,L}$ may contribute to the AP-plateau in another way in aged than in young hearts.

Changes in $I_{Ca.L}$

An increase in absolute $I_{Ca,L}$ amplitude during aging as the cells become enlarged has been reported various times (for review see (6, 7) mainly in rats. However, if the $I_{Ca,L}$ amplitude is normalised to the cell capacitance it remains constant (4, 5, 22) During further progression to severe hypertrophy or congestive heart failure $I_{Ca,L}$ density seems to be reduced (6). This is consistent with our results in a rabbit model, which showed that L-type channel maximal conductance, was significantly reduced in aged hearts.

To calculate the current density, the current amplitude has been normalised to the membrane surface to correct the current for different cell size, which is a classical standard. However, if excitation-contraction coupling is concerned, normalisation to cell volume is preferable. Satoh and coworkers (23) showed that the membrane capacity is a good measure for the cell volume. In rabbit myocytes they recorded a volume of 30 pl per cell and a capacity/volume ratio of 4.58 pF/pl in good accordance with our results obtained in young animals (cell volume: 23.6 pl; cell capacity: 108.0 pF, ratio capacity/volume 4.57pF/pl). With increasing age cardiomyocytes develop hypertrophy. In rats ventricular cardiomyocytes grow mainly in longitudinal direction (5, 23). To our knowledge this distinctive growth pattern has not been reported in a rabbit model yet. In contrast, in the case of perinephritis-induced hypertension ventricular myocytes of rabbits grew in lenght and width (24), which is consistent with our observations. Growth in two dimensions shown by McIntosh and coworkers (24) was present even though the hypertrophy gained in their study was smaller than in ours (increase in cell capacity by a factor of 1.5 *vs*. 2.5 here). In rats the capacity/volume ratio changes during development, three month old animals exhibit 6.76 pF/pl and 6 month old 8.88 pF/pl (23). This can be interpreted as a compensation mechanism for a developing hypertrophy. Such a mechanism has not been shown in rabbits and was not observed here (young rabbits: 4.57 pF/pl *vs*. aged rabbits: 3.38 pF/pl). The diminished surface to volume ratio in cells from aged rabbits enhances the influence of the reduction of $I_{Ca,L}$, thus the $I_{Ca,L}$/volume ratio becomes significantly smaller ($p<0.001$) during aging from 46.6±1.38 pA/pl to 29.52±2.44 pA/pl, *i.e*. by 36.6%.

However, a reduced peak Ca^{2+} influx might be compensated by a prolongation of current flow as shown in rat ventricular cells (5). In rabbit ventricular cells the time constants of inactivation τ_1 and τ_2 remained unchanged (see results). Thus, this compensation mechanism does not seem to be effective here.

Changes in action potential duration

A prolongation in APD is able to enhance the late component of $I_{Ca,L}$ during the AP plateau. This component of $I_{Ca,L}$ is relatively important in rabbit and guinea-pig ventricular cardiomyocytes, but less important in rat cells (25). Thus, a reduced peak Ca^{2+} influx could be compensated by a prolonged late component of $I_{Ca,L}$. In fact a prolongation of the APD due to hypertrophy has been reported various times (review (3)) and was indicated in our study. Also the shift of the inactivation curve to more positive potentials supports an enhanced late component of $I_{Ca,L}$. As the late component of $I_{Ca,L}$ controls loading of the sarcoplasmic reticulum, SR, with Ca^{2+} (26) an enhanced late component of $I_{Ca,L}$ will induce a higher loading of the SR which will increase the role of SR calcium release in excitation-contraction coupling. It can be suggested that an enhanced late component of $I_{Ca,L}$ might help to counterbalance the reduced peak current per volume.

These considerations are also consistent with the observed effects of verapamil. In the aged hearts verapamil caused a significantly stronger shortening of the ARI as well as a weaker reduction in LVP. The initially prolonged ARI in aged heart under control conditions could be due to altered potassium currents in the older animals. Thus, in aged rat hearts prolonged APD has been attributed to altered I_{to}, while I_{K1} was un-affected (4, 5). While in rabbits I_{to} is less abundant than in rats, a reduction of most other K^+-current densities was seen in hypertrophied rabbit heart (24). The observation that verapamil caused ARI shortening in aged but not in young hearts may be theoretically supported by a side effect of verapamil on $I_{K.r}$ (27). If $I_{K.r}$ channels are less abundant in older myocytes, their block might prolong repolarisation less than in younger animals. Then, the block of L-type channels would be more important for APD resulting in ARI shortening. However, the concentration range at which verapamil exerts its effect on $I_{K.r}$ has to be discussed: in HEK 293 cells HERG-encoded $I_{K.r}$ channels were blocked by verapamil with an IC_{50} of 0.147 µM/L (27) or 0.830 µM/L in COS cells (28), while in our study 0.05 µM/L were used. Thus, such an effect on $I_{K.r}$ probably plays a minor role in our study. In smooth muscle cells it was shown that reduced activity of Na^+/Ca^{2+}-exchanger can result in increased K^+ currents by enhanced sensitivity of large-conductance Ca^{2+}-activated K (BK_{Ca}) channels (29). However, in heart BK_{Ca} channels are not expressed (30).

β-adrenergic augmentation of $I_{Ca,L}$ was not significantly depressed in our recordings. In rat (22) and human (31) the response to β-adrenergic stimuli decreases during progredient hypertrophy. We assume preservation of β-adrenergic augmentation of $I_{Ca,L}$ as a sign of only moderate hypertrophy in our cells. The observed age-dependent decrease of carbachol effect on isoprenaline-stimulated $I_{Ca.L}$ is in good agreement with other observations of a reduction of muscarinic receptor density with age (32).

Another consequence of the increase in cell volume is that the nucleus/cytoplasm ratio is markedly reduced during aging which has also been reported in rats (33).

Changes in calcium handling proteins and inotropy

The expression of the three different proteins characterizing the calcium homeostasis in young and old subjects, α1c subunit of the L-type channel, the SERCA2a-ATPase, and the Na^+/Ca^{2+} exchanger did not differ significantly between the two age groups. Although earlier studies demonstrated the regulation of these proteins in rabbit heart (33), we failed to determine a significant change of expression between old and young animals. However, the atrial expression of α1c subunit of the L-type channel and the Na^+/Ca^{2+} exchanger seemed lower compared to the ventricular expression, but they did not differ significantly. A lower expression of SERCA2a-ATPase in rabbit ventricle than in atrium has already been reported (34). Taken together, changes found in the electrophysiology of the cardiomyocytes studied were not associated with significant changes of expression of proteins involved in calcium homeostasis. With respect to our results we cannot exclude, however, an altered activation potential of these proteins due to posttranscriptional and postranslational modifications.

If verapamil-effects on APD are pronounced and $I_{Ca.L}$/cell volume is reduced, one might ask, whether more pronounced negative inotropic effects can also be expected. However, since baseline inotropy was similar between young and aged hearts, reduced $I_{Ca.L}$/cell volume-ratio must be compensated by another source of calcium, probably by enhanced release from the SR, as was observed in cardiomyocytes from failing hearts or in cardiomyocytes treated with FK-506 (35). In that case, the negative inotropic effect of verapamil (exerted *via* $I_{Ca.L}$ blockade) should be decreased, since under these conditions the release from SR (or other sources) would be more important. In accordance with this consideration, the negative inotropic effect of verapamil was indeed reduced in aged hearts in this study. Another point, which may alter the verapamil effect in aged hearts due to the prolonged action potential duration together with later inactivation of $I_{Ca.,L}$, is that verapamil binding to $I_{Ca,L}$ is state-dependent with binding of the drug to the inactivated state at more positive potentials (36).

Finally, data on QRS support our previous finding, that QRS duration is prolonged in aged hearts which has been interpreted as a consequence of conduction slowing by collagen deposition (2). Furthermore, our present data show that verapamil does not affect QRS and, thus, probably does not affect ventricular conduction properties. The data on VEC furthermore support our previous finding (2) showing that two succeeding heart beats exhibit more beat to beat variability in aged hearts with lower similarity of activation patterns for two succeeding heart beats, while verapamil exhibited similar effects in both groups.

CONCLUSIONS

According to the classification by (6) the hearts investigated here had not yet entered the stage of severe hypertrophy, but were still in the stage of moderate hypertrophy. However, certain biophysical changes, necessary to compensate the hypertrophy of the cells may help to explain the altered pharmacodynamic effect of verapamil inducing ARI-shortening and attenuated negative inotropy in aged hearts. We conclude, that there are distinct alterations in the electrophysiology of $I_{Ca.L}$ in the aged heart which may influence the response to verapamil in a complex manner. The reduced $I_{Ca.L}$/cell volume ratio and reduced maximum conductance in aged hearts seem to be compensated by a positive shift of the steady state inactivation, thereby enhancing the late component of $I_{Ca,L}$, which might enhance loading of the SR. This -besides the other factors discussed above- could reduce the negative inotropic effect of verapamil. These results demonstrate that the pharmacodynamics of a certain drug may be altered in aged hearts.

Since *e.g.* the hypothalamic-pituitary-adrenal axis regarding CRH- or β_2-adrenoceptor-dependent ACTH-secretion is also controlled by L-type Ca^{2+} current (37), it might be interesting to investigate age-dependent $I_{Ca.L}$ function and the effects of $I_{Ca.L}$ blockers in other organs in future studies.

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