Effect of tachycardia on myocardial sarcoplasmic reticulum and Ca^{2+} dynamics: a mechanism for preconditioning?

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Abstract

We have previously demonstrated that brief episodes of tachycardia prior to a prolonged occlusion of a coronary artery, followed by reperfusion, substantially reduce the infarct size. Adenosine receptors and mitochondrial ATP-dependent K^+ channels mediate this effect. Since preconditioning can be induced or reverted by maneuvers that increase or decrease $[Ca^{2+}]_i$, respectively, and tachycardia increases $[Ca^{2+}]_i$, we studied the participation of sarcoplasmic reticulum and Ca^{2+} in the preconditioning effect of tachycardia. We measured the effect of ischemia and tachycardia on Ca^{2+} uptake and release by sarcoplasmic reticulum vesicles isolated from left ventricular canine myocardium. Myocardial ischemia increased Ca^{2+}-release rate constants and decreased both the initial rates of Ca^{2+} uptake and $[^3H]$-ryanodine binding by sarcoplasmic reticulum. In addition, ischemia induced a decrease in the pentameric form of phospholamban and in the content of ryanodine-receptor Ca^{2+}-release channel protein. All these effects were reverted in hearts preconditioned with tachycardia. Furthermore, tachycardia by itself increased $[^3H]$-ryanodine binding, Ca^{2+}-release rate constants and the protein levels of ryanodine-receptor Ca^{2+}-release channels and the ATP-dependent Ca^{2+} pump. These results suggest that tachycardia preserves the integrity of the sarcoplasmic reticulum preventing the excess of release and the decrease of uptake of Ca^{2+} produced by ischemia, thereby avoiding cytosolic Ca^{2+} overload. This sarcoplasmic reticulum protection could partly explain the preconditioning effect of tachycardia.

Keywords: Preconditioning; Tachycardia; Ca^{2+}; Sarcoplasmic reticulum; Myocardial Infarction

1. Introduction

Myocardial ischemic preconditioning with brief episodes of ischemia induces early and late protection against the effects of a prolonged subsequent ischemia [1]. This protection involves a decrease of infarct size, incidence of arrhythmias [2,3] and contractile dysfunction [4]. The early protection seems to be mediated by activation of adenosine receptors, nitric oxide synthesis, protein kinases (PK) like PKC and tyrosine kinase, and probably by the activation of mitochondrial ATP-dependent K^+ (K^+_{ATP}) channels as the end effector [5]. We recently demonstrated that several episodes of tachycardia, instead of ischemic episodes, induce early preconditioning against infarct size [6], an effect that is mediated by mitochondrial K^+_{ATP} channels [7].

Preconditioning can be induced by maneuvers that increase $[Ca^{2+}]_i$, like the intracoronary administration of $CaCl_2$ [8,9], or reverted by maneuvers that decrease $[Ca^{2+}]_i$, like blockade of L-type Ca^{2+} channels [10,11]. As tachycardia induces a transient $[Ca^{2+}]$ increase in the cytosol [12,13], we decided to investigate the participation of the sarcoplasmic reticulum (SR) and Ca^{2+} in the preconditioning effect induced by tachycardia in dogs. Our results show that ischemia produces an increase in the Ca^{2+}-release rate constants, a decrease in the initial rate of Ca^{2+} uptake by the SR and a decrease in the protein levels of ryanodine-receptor Ca^{2+}-release channels (RyR2) and pentameric phospholamban (PLB). Tachycardia produced an early increase in Ca^{2+}-

Abbreviations: C, control dogs; I, non-preconditioned dogs with myocardial ischemia; K^+_{ATP}, ATP-dependent K^+ channels; PK, protein kinases; PLB, phospholamban; RyR2, cardiac ryanodine-receptor Ca^{2+}-release channels; SERCA2, Ca^{2+} ATP-dependent SR pump; SR, sarcoplasmic reticulum; T, group of dogs treated with tachycardia; T + I, group of ischemic dogs preconditioned with tachycardia.

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release rate constant and reverted the alterations of SR function induced by ischemia.

2. Materials and methods

2.1. Experimental design

We used the same preparation as in previous experiments in which the preconditioning effect of tachycardia on infarct size was observed [6]. Fifty-six dogs were anesthetized with sodium pentobarbital (30 mg/kg, i.v.), the thorax was opened and the arteriovenous conduction was blocked by the injection of formaldehyde in the upper interventricular septum. The heart rate was controlled with an electrical stimulator through electrodes implanted in the outflow tract of the right ventricle. Sylastic catheters were implanted into the root of the aorta and in the great coronary vein for pressure measurements and blood sampling. A flow probe (Nihon Kodhen) was implanted on the anterior descending coronary artery, distal to the first diagonal branch, and a plastic snare was implanted below the probe. To prevent from large changes in mean aortic pressure during the experiments, a damping system was set up by connecting the systemic circulation, with a wide bore catheter implanted in a femoral artery, to a reservoir filled with homologous, thermoregulated and constantly stirred blood. The altitude of the reservoir was adjusted to maintain a mean pressure at about 90 mmHg in the root of the aorta [6].

Five dogs died during surgery. After stabilization of the preparation (about 10 min) the remaining 51 dogs were randomly allocated to the following groups (Fig. 1):

1. Control dogs (C, $n = 13$): After 50 min at a heart rate of about 120 c/min the heart was excised and transmural samples were obtained from the left ventricular wall for analysis.
2. Ischemia in non-preconditioned dogs (I, $n = 13$): Similar to group C but after 50 min at a heart rate of 120 c/min the anterior descending coronary artery was occluded for 50 min and then the heart was excised and samples were obtained from the ischemic region demarked by a cyanotic color.
3. Ischemia in preconditioned dogs (T + I, $n = 13$): Similar to group I, but five periods of tachycardia at 210 c/min, 5 min in duration each, with intervening periods of 5 min at basal heart rate (120 c/min) were induced before occluding the coronary artery for 50 min and then the heart was excised and samples obtained as in group I.
4. Dogs only with tachycardia (T, $n = 12$): Similar to group C, but without ischemia. The heart was excised and samples were obtained immediately after the last period of tachycardia. This group was designed in order to observe the early changes induced by tachycardia.

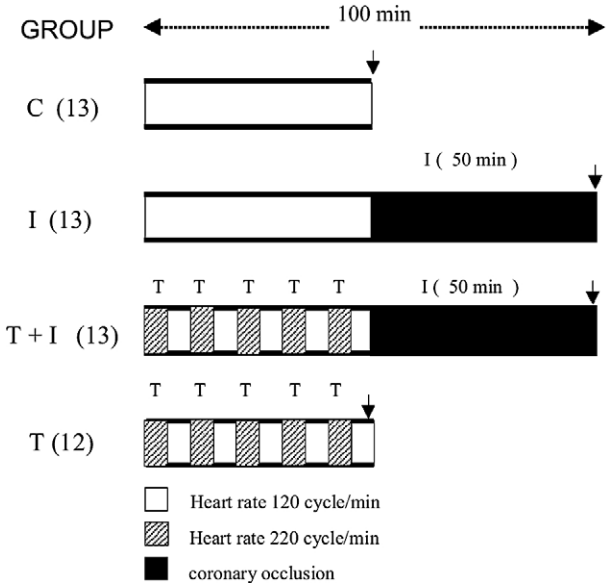

Fig. 1. Experimental protocol: C, control; I, ischemia; T + I, ischemia preceded by episodes of tachycardia; T, brief episodes of tachycardia. The arrow indicates the point at which hearts were removed for preparing SR. In parenthesis number of dogs for each experimental condition.

Coronary arteriovenous difference in O_2 and lactate content were measured during the episodes of tachycardia and after that when aortic pressure had returned to baseline in groups with tachycardia (groups T and T + I) and at equivalent times in groups without tachycardia (groups C and I). Myocardial O_2 consumption was calculated as the product of coronary blood flow, measured with the flowmeter, times the coronary arteriovenous difference in O_2 content. Aortic pressure and the ECG were continuously monitored.

2.1.1. Exclusion criteria

In order to avoid differences in the degree of myocardial ischemia between groups, dogs in which more that two attempts for electrical defibrillation were needed were discarded.

2.2. Isolation of SR vesicles

SR vesicles were isolated from canine hearts with the method described by Inui et al. [14] and modified by Sánchez et al. [15]. Briefly, hearts were perfused through the aorta with a cold cardioplegic solution (in mM): 2 $CaCl_2$, 16 $MgCl_2$, 102 NaCl, 20 KCl, 2 EGTA, 20 MOPS adjusted with Tris base to pH 6.8. All the following procedures were carried out at 4 °C. The left ventricle was finely minced and homogenized in four volumes of ice-cold solution which contained: 300 mM sucrose, 20 mM MOPS adjusted with Tris base to pH 6.8, and a combination of protease inhibitors (4 μg/ml leupeptin, 4 μg/ml pepstatin A, 1 mM benzamidine and 1 mM PMSF). Tissues were homogenized in a Heidolph Diax 600 homogenizer using three 15-s bursts at 24,000 rpm, each burst was followed by 5-s rest intervals. The homogenate was sedimented at 3800 × g for 15 min and the resulting pellet was homogenized in three volumes of homogenization buffer as above, and sedimented at 3800 × g for 15 min. The combined supernatants were filtered through cheesecloth and sedimented at 28,000 × g for 15 min. Solid KCl was added to

the supernatant to a final concentration of 0.65 M before centrifugation at 120,000 × *g* for 1 h. The pellet, enriched in SR vesicles, was resuspended in homogenization buffer to a final protein concentration of 10 mg/ml, fractionated in small aliquots and snap frozen in liquid N_2. Vesicles were stored at – 80 °C for up to 2–3 weeks.

SR vesicles were obtained from each heart, in all groups, and the following studies were performed in each one of them. In some determinations the whole homogenate, filtered through four layers of cheesecloth, was used without any further fractionation.

2.3. Oxalate-supported Ca^{2+} uptake

The initial rate of oxalate-supported Ca^{2+} uptake was determined by incubating the vesicles (0.05 mg/ml) at 37 °C in a solution containing (in mM): 150 KCl, 0.1 $^{45}CaCl_2$ (specific activity 10–20 mCi/mmol), 0.1 EGTA, 5 $MgCl_2$, 5 Na_2ATP, 5 potassium oxalate, 5 NaN_3, 0.025 ruthenium red and 20 MOPS–Tris, pH 6.8. Fractions (0.2 ml) were removed at different times and filtered through Millipore filters (HA. 0.45 M). Filters were washed twice with 3 ml of ice-cold non-radioactive incubation solution without ATP. The amount of ^{45}Ca retained in the filters was determined in a liquid scintillation counter.

2.4. Ca^{2+}-release kinetics

The Ca^{2+}-release kinetics from isolated SR vesicles actively loaded with Ca^{2+} was measured in an SX.18MV fluorescence stopped-flow spectrometer from Applied Photophysics Ltd. (Leatherhead, UK) as previously described [16]. Isolated SR vesicles (1 mg/ml) were actively loaded with Ca^{2+} at 25 °C by incubation in a solution containing (in mM): 0.05 $CaCl_2$, 100 KCl, 10 phosphocreatine, 15 U/ml creatine kinase, 20 imidazole–MOPS, pH 7.2. Ca^{2+}-release through RyR2 channels was induced by mixing one volume of actively loaded vesicles with 10 volumes of a solution containing (in mM): 100 KCl, 1.6 ATP, 1 μM calcium Green 5 N, 20 imidazole–MOPS; pH 7.2. After mixing, this solution produces 1.2 mM free [ATP] and pCa 6.

Since SR vesicles from the different experimental groups transported Ca^{2+} at different rates, we determined in each case the incubation time necessary to load all the extravesicular Ca^{2+} added. For this purpose, we measured the decrease in extravesicular free [Ca^{2+}] in a fluorescence spectrophotometer (FluoroMax-2; JOBIN YVON-SPEX) as described [15]. Uptake was initiated by adding a small volume of $MgCl_2$ and ATP (final concentration 3 and 2 mM, respectively) and it was completed (i.e. [Ca^{2+}] in the cuvette decreased to 0.3–0.4 μM) in <20 min in SR vesicles from control dogs and dogs treated with tachycardia. Longer time (30–40 min) was required to take up all the extravesicular Ca^{2+} in vesicles derived from ischemic animals but the final [Ca^{2+}], measured with calcium Green 2 N, was similar to controls and <0.4 μM. After cessation of uptake, all the vesicles retained the accumulated Ca^{2+} for periods ≥30 min.

2.5. [^{3}H]-ryanodine binding

Isolated SR vesicles (0.1 mg/ml) or whole homogenates (0.5 mg/ml) were incubated with 10 nM [^{3}H]-ryanodine in 0.15 M KCl at pCa 5 as previously described [17].

2.6. Western blot assays

Isolated SR vesicles were solubilized in sample buffer at 95 °C for 5 min and subjected to SDS-PAGE (3–8% gradient for RyR2 and 7% for Ca^{2+} ATP-dependent pump, SERCA2) under reducing conditions. Non-reducing conditions SDS-PAGE (15%) were used for PLB. Proteins were transferred to PVDF membranes and probed with primary antibodies against SERCA2 (MA3-919), PLB (MA3-922) and RyR2 (MA3-916) (Affinity Bioreagents Inc., diluted 1:2000 in phosphate-buffered saline, PBS). After washing, membranes were incubated with secondary antibodies (IgG) conjugated to horseradish peroxidase (SA1-100) (1:5000 dilution in PBS). Protein–antibody reactions were detected by chemiluminescence using Kodak BioMax films. The relative amount of proteins on the blots was determined by densitometric analysis using an SNAP-SCAN 1212P scanner (AGFA) and Quantity One software (Bio-Rad). Vesicles isolated from two controls, one tachycardic, one ischemic and one preconditioned dogs were run in each gel, and care was taken to load exactly the same amount of total protein for each one of them. The total protein loaded in gels to detect PLB was 10 μg; 20 μg were loaded for SERCA2 and 30 μg for RyR2. Routinely, two gels were loaded in the same conditions and were run in parallel; one was stained with Coomassie Blue to check for protein load and the other one was transferred. In some experiments protein load was also checked by Ponceau Red staining of the transferred proteins. Samples were run in duplicate and results were expressed as percentage of the same protein band present in the control dog. Results obtained from the same samples were analyzed in different gels and in different days differed less than 10%.

2.7. Protein determination

It was performed according to Hartree [18], using commercial bovine serum albumin as standard.

All reagents used were of analytical grade. Protease inhibitors were obtained from Sigma Chemical Co. [^{3}H]-ryanodine and $^{45}CaCl_2$ were obtained from DuPont-New England Nuclear Corp.

2.8. Statistical analysis

Comparison of hemodynamic and metabolic variables, Ca^{2+} uptake and release and ryanodine-binding changes were performed with ANOVA followed by Student–Knewman–Keuls' analysis. Results are expressed as mean ± S.E. The null hypothesis was discarded with a *P*-value <0.05.

Table 1
Hemodynamic and metabolic changes

Group	*N*	Maneuver	HR (c/min)	MAP (mmHg)	CF (ml/min/100 g)	MQO_2 (ml/min/100 g)	$(A\text{-}V)O_2$ (ml/100 ml)	VO_2 (ml/100 ml)	(A-V) lactate (μmol/ml)
C	13	B	119 ± 4.9	93.1 ± 7.1	73.0 ± 5.9	6.9 ± 0.6	9.3 ± 0.2	9.16 ± 0.3	0.47 ± 0.02
I	10	B	115 ± 4.6	88.9 ± 2.0	71.0 ± 4.2	6.7 ± 0.3	9.6 ± 0.5	8.80 ± 0.2	0.48 ± 0.02
		I	118 ± 4.5	84.4 ± 2.5	N/A	N/A	N/A	N/A	N/A
T + I	11	B	122 ± 4.0	93.5 ± 2.6	69.3 ± 4.4	7.2 ± 0.4	10.7 ± 0.5	8.6 ± 0.4	0.51 ± 0.03
		T	214 ± 2.8 *	91.0 ± 2.8	98.4 ± 2.6 *	11.6 ± 0.5 *	11.9 ± 0.4 *	6.5 ± 0.3 *	0.56 ± 0.02 *
		I	118 ± 4.2	88.0 ± 2.5	N/A	N/A	N/A	N/A	N/A
T	12	B	116 ± 4.8	95.0 ± 2.5	65.4 ± 4.1	6.9 ± 0.6	10.4 ± 0.2	8.2 ± 0.3	0.53 ± 0.07
		T	215 ± 2.0 *	89.2 ± 3.0	95.2 ± 4.0 *	11.2 ± 0.5 *	12.0 ± 0.2 *	6.6 ± 0.2 *	0.61 ± 0.02 *

HR, heart rate; MAP, mean arterial pressure; CF, coronary flow; MQO_2, myocardial oxygen consumption; $(A\text{-}V)O_2$, coronary arteriovenous oxygen content difference; VO_2, coronary venous oxygen content; (A-V) lactate, coronary arteriovenous lactate concentration difference. C, control; I, ischemia; T + I, myocardial ischemia preceded by tachycardia; T, tachycardia.
* $P < 0.05$ (at least) vs. baseline B, baseline.

3. Results

Of the 51 dogs allocated to the four groups, three dogs were discarded in the group treated with ischemia (I) and two in the group with tachycardia followed by ischemia (T + I), because more than two attempts for electrical defibrillation were needed in each case.

3.1. Hemodynamic and metabolic changes produced by tachycardia

Table 1 shows the hemodynamic and metabolic variables in all the groups. Mean aortic pressure did not change significantly between groups. During tachycardia (groups T + I and T) myocardial O_2 consumption and coronary blood flow increased by about 64% and 44%, respectively, as compared to groups without tachycardia and to the basal values in the corresponding group. However, the coronary arteriovenous O_2 difference did not increase by more than 13%; the coronary sinus venous O_2 content did not decrease by more than 22% and the coronary arteriovenous difference in lactate content increased at least by 8%. The ECG did not show repolarization changes suggesting ischemia.

All the biochemical analysis were performed in 26 of the 46 dogs. In the other 20 dogs, six controls (C), four with ischemia (I), four with tachycardia plus ischemia (T + I) and six only with tachycardia (T), the only biochemical analysis performed was [^{3}H]-ryanodine binding in the whole homogenate (0.5 mg/ml) in order to compare these results with those obtained in isolated SR vesicles.

3.2. Analysis of isolated SR vesicles (n = 26)

3.2.1. Initial rate of oxalate-supported Ca^{2+} uptake

The isolated SR vesicles used in this work showed active Ca^{2+}-uptake activity which was completely inhibited by 1.5 μM thapsigargin, a specific inhibitor of SERCA2 and it was not modified by 5 mM sodium azide, indicating that the uptake was not due to contaminating mitochondrial membranes (Fig. 2A). We determined the initial rate of oxalate-supported active Ca^{2+} uptake in order to compare the activity of SERCA2 in the different experimental groups. In control animals the initial rate of Ca^{2+} uptake was 91 ± 4 nmol/mg protein/min ($n = 6$) (Fig. 2B). Ischemia decreased this uptake to 28 ± 1 nmol/mg protein/min ($n = 6$; $P < 0.001$) an effect that was reverted in SR vesicles isolated from animals in which tachycardia was induced previous to ischemia

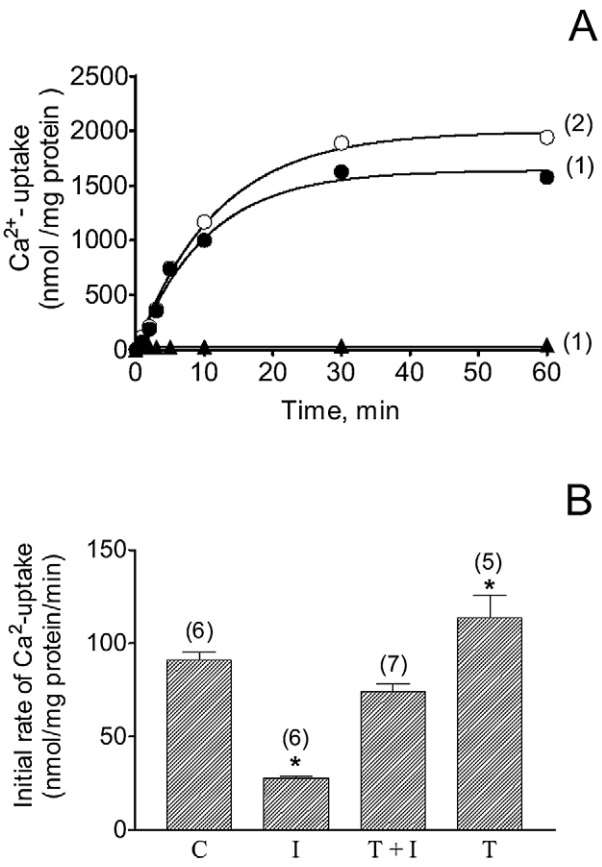

Fig. 2. (A) Time course of oxalate-supported active Ca^{2+} uptake in isolated SR vesicles: control (open circles); 5 mM sodium azide (closed circles); 1.5 μM thapsigargin (closed triangles). Uptake conditions as indicated in Section 2. (B) Initial rate of oxalate-supported active Ca^{2+} uptake in the different experimental protocols: C, control; I, ischemia; T + I, ischemia preceded by tachycardia; T, tachycardia alone. Uptake conditions as indicated in Section 2. Bars represent the mean ± S.E. * $P < 0.05$ vs. control. In parenthesis number of hearts for each experimental condition.

(74 ± 4 nmol/mg protein/min; n = 7). Tachycardia alone increased significantly the initial rate of Ca^{2+} uptake to 114 ± 12 nmol/mg protein/min as compared to control (n = 5; P < 0.05) (Fig. 2B). In contrast, initial rates of Ca^{2+} uptake in the ischemic zone of hearts preconditioned with tachycardia and in the non-ischemic region of the ischemic hearts were not significantly different from control (not shown).

3.2.2. Fast Ca^{2+}-release kinetics

Isolated vesicles were actively loaded with Ca^{2+} in the presence of an ATP-regenerating system and without the addition of potassium oxalate or other inhibitors that would interfere with Ca^{2+} release. When vesicles (1 mg/ml) were incubated in 50 μM $CaCl_2$ the amount of Ca^{2+} loaded was on average 40 ± 2 nmol Ca^{2+}/mg protein and was not different among the four experimental groups. Vesicles released 50–70% of the accumulated Ca^{2+} when mixed with the releasing solution that maximally stimulated Ca^{2+} release, without significant differences between the experimental groups (data not shown). This release of Ca^{2+} was inhibited by ruthenium red, a specific inhibitor of RyR2/Ca^{2+}-release channel.

Fast-release kinetics was determined by measuring the increase in fluorescence of the Ca^{2+} indicator calcium Green 5 N over a millisecond time scale in a stopped flow fluorescence spectrophotometer. Typical records are shown in Fig. 3. Ca^{2+} release followed double exponential kinetics with a fast (k_1)- and a slower (k_2)-release components, each contributing 50% to the total fluorescent change. The rate constant of the k_1 was significantly higher in ischemic than in control hearts (222 ± 14 s^{-1} (n = 5) vs. 101 ± 4 s^{-1} (n = 6), P < 0.001). Preconditioning with tachycardia reverted the effect of ischemia and decreased k_1 to 130 ± 5 s^{-1} (n = 5), a value not significantly different from control. Tachycardia alone significantly increased k_1, to 163 ± 15 s^{-1}, compared to control (n = 6, P < 0.01) (Fig. 3). No significant changes among the groups were observed for k_2.

3.2.3. [^{3}H]-ryanodine binding

3.2.3.1. Measurements in isolated SR vesicles. Ischemia decreased [^{3}H]-ryanodine binding by 48%, from the control value of 3.1 ± 0.1 (n = 7) to 1.6 ± 0.1 pmol/mg protein (n = 6) (P < 0.01) (Fig. 4). This decrease was prevented in animals preconditioned with tachycardia (2.6 ± 0.2, n = 7). Tachycardia itself significantly increased [^{3}H]-ryanodine binding to 5.2 ± 0.5 pmol/mg protein (n = 6), which represents a 68% increase compared to control (P < 0.001).

3.2.3.2. Measurements in whole homogenates (n = 20). [^{3}H]-ryanodine binding in whole homogenates showed similar results to those obtained in isolated SR vesicles. Ischemia reduced [^{3}H]-ryanodine binding by 42%, from the control value of 0.166 ± 0.01 pmol/mg protein (n = 6) to 0.104 ± 0.016 pmol/mg protein (n = 4) (P < 0.01). Preconditioning with tachycardia (n = 4) completely reverted the effect of ischemia to 0.188 ± 0.008 pmol/mg protein, a value not significantly different from control. Tachycardia alone (n = 6) increased binding by 61% to a value of 0.290 ± 0.009 pmol/mg protein (P < 0.001) (Fig. 4).

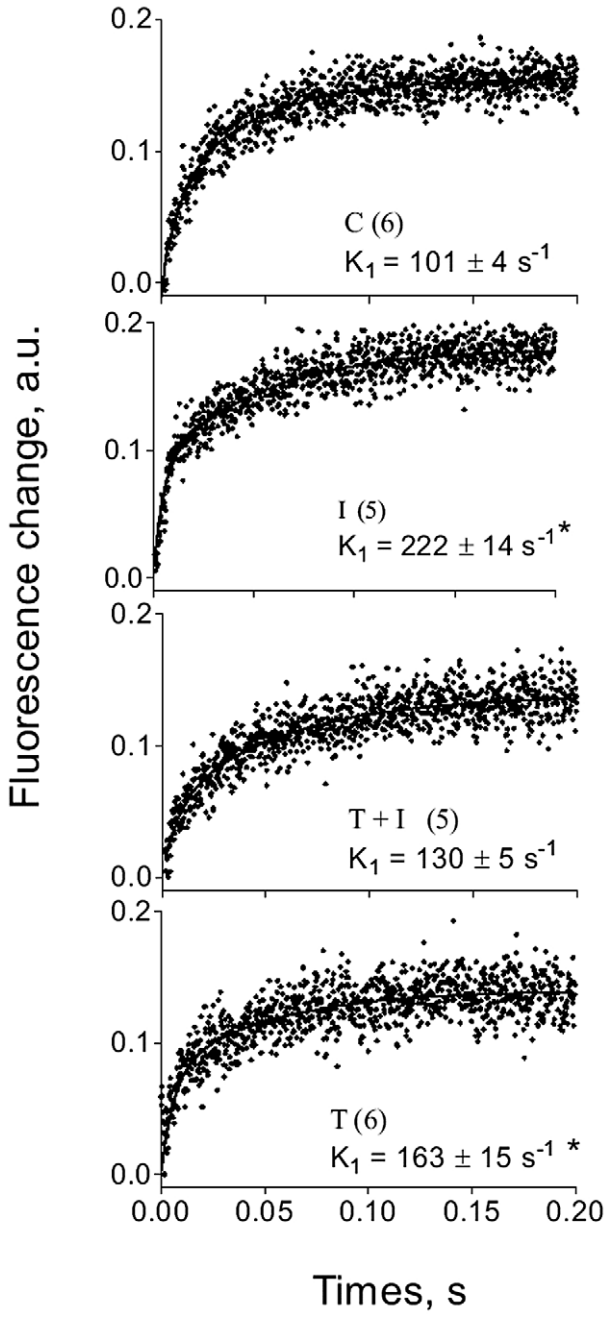

Fig. 3. Ca^{2+}-release records in isolated SR vesicles in the different experimental protocols: C, control; I, ischemia; T + I, ischemia preceded by tachycardia; T, tachycardia alone. The rate constant of the fast-exponential release (k_1) is given as mean ± S.E. * P < 0.001 vs. respective control. In parenthesis number of hearts for each experimental condition.

3.2.4. Western immunoblot analysis of SR proteins

The protein content of RyR2, SERCA2 and PLB was determined by western blot. Fig. 5A shows a typical result comparing protein content in the different experimental pro-

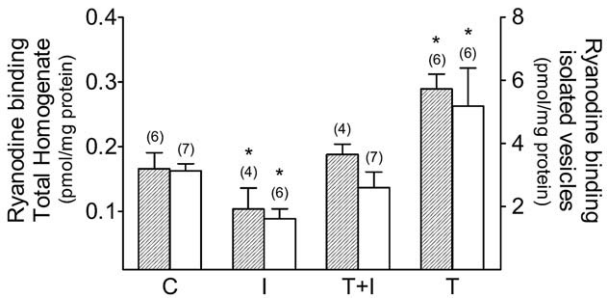

Fig. 4. [^{3}H]-ryanodine binding in whole homogenate (filled bars) and in isolated SR vesicles (empty bars) in the different experimental protocols: C, control; I, ischemia; T + I, ischemia preceded by tachycardia; T, tachycardia alone. Bars represent the mean ± S.E. * $P < 0.01$ vs. control. In parenthesis number of hearts for each experimental condition.

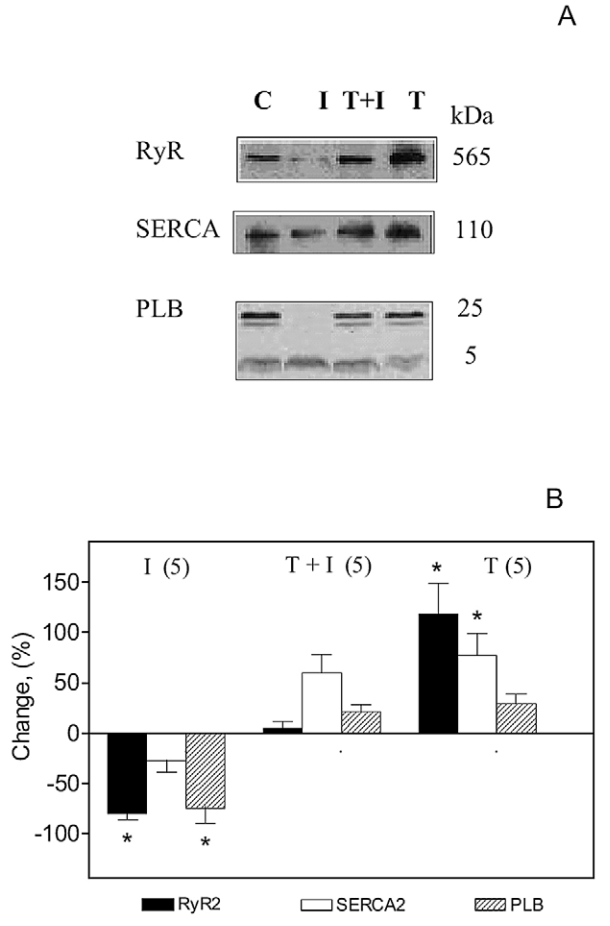

Fig. 5. (A) Typical western blot analysis of RyR2, SERCA2 and PLB in C, control; I, ischemia; T + I, ischemia preceded by tachycardia; T, tachycardia alone. (B) Percent change protein content of RyR2, SERCA2 and PLB in the different experimental protocols. Results are expressed as the average of the relative change of densitometric units with respect to control ± S.E. * $P < 0.01$ vs. control. In parenthesis number of hearts for each experimental condition.

tocols. Fig. 5B shows the protein content in the different protocols. Ischemia ($n = 4$) decreased the protein content of RyR2 and the pentameric PLB in SR membranes both by 80% ($P < 0.01$). Most of the decrease in the pentameric form of PLB (25 kDa) produced by ischemia could be accounted for an increase in the monomeric form (Fig. 5A). The protein content of SERCA2 was not significantly modified by ischemia. Preconditioning with tachycardia prevented the decrease in pentameric PLB and RyR2 proteins produced by ischemia and increased SERCA2 protein content by 60% as compared to control. Tachycardia alone increased both RyR2 and SERCA2 content by 120% and 80%, respectively, as compared to control, but did not modify the content of pentameric PLB.

4. Discussion

Our results show that myocardial ischemia increases the rate constant of Ca^{2+} release and decreases the rate of Ca^{2+} uptake by the SR. Both effects could contribute to produce a cytosolic Ca^{2+} overload with the consequent damage of the cell. Preconditioning with tachycardia prevented these effects of ischemia. In addition, the alterations produced by ischemia on SR Ca^{2+} fluxes were associated to a decrease in protein levels of RyR2 and pentameric PLB, and these effects were also reverted by preconditioning with tachycardia. Finally, an increase in Ca^{2+}-release rate constant was observed immediately after tachycardia. It is worth noticing that these protective effects of tachycardia are not due to ischemic preconditioning since tachycardia increased myocardial O_2 consumption without producing ischemia. This is revealed by the small observed changes in coronary arteriovenous O_2 difference and venous O_2 content, which are similar to those reported during exercise in well-trained conscious dogs [19]. Besides, there was an increase in lactate extraction by the heart and no ischemic alterations were observed in the ECG. These results agree with those that we previously reported in a similar preparation to demonstrate the non-ischemic preconditioning effect of tachycardia [6] and of exercise [20].

In this work we used isolated SR vesicles partially purified to study SR Ca^{2+} transport because the SR proteins responsible for Ca^{2+} handling, specially the ryanodine receptor, are minor components of the total heart proteins. Thus, working with a whole homogenate would make it difficult to correlate the obtained results with the intrinsic properties of the protein of interest. Moreover, it has been shown that the properties of the isolated SR vesicles closely mimic the properties of whole homogenates in regard to Ca^{2+} release [21] or [^{3}H]-ryanodine binding [22]. In this regard, our [^{3}H]-ryanodine-binding results obtained in the homogenates were similar to those obtained for the purified SR fraction. Furthermore, a low protein concentration is needed for fast-release kinetics determinations in a stopped flow fluorescence spectrometer, otherwise a high protein content and the presence of large particles in a crude homogenate will extensively quench the fluorescent signals.

Our results agree with those of Wu and Feher [23] who found that ischemia produced a decrease in SR Ca^{2+} uptake, both in homogenates and in isolated SR vesicles from isolated perfused rat hearts. However, these authors found, at variance with us, a small increase in ryanodine binding in the

case of isolated SR. Darling et al. [24] observed a decrease in Ca^{2+} uptake by canine myocardial homogenate and SR vesicles by effect of hypoxia, although the effect was similar in the presence or absence of ryanodine. Furthermore, these authors did not find a substantial loss of intrinsic SR Ca^{2+}-release channel function nor of its structural integrity. However, in these experiments [24] in vitro hypoxic but not ischemic myocardium was compared to normal tissue. On the other hand, several authors [25–27] using isolated SR membrane fractions have reported an impairment of SR Ca^{2+} uptake and Ca^{2+} ATPase activity by effect of ischemia. Thus, Osada et al. [27] reported that ischemia sustained for 20 min produces a marked depression in SR Ca^{2+}-release and -uptake activities as well as SR Ca^{2+}-pump protein content, alterations that were significantly attenuated by ischemic preconditioning. Furthermore, Temsah et al. [28] reported in the isolated rat heart that ischemia–reperfusion and the resulting induced Ca^{2+} paradox produce a marked decrease in mRNA levels for the SR proteins, an effect that was attenuated by ischemic preconditioning. These results [27,28] are analogous to our results obtained with preconditioning with tachycardia instead of preconditioning with ischemia. We observed that ischemia in the absence of preconditioning with tachycardia decreased ryanodine binding although the rate constant of Ca^{2+} release increased. This apparently paradoxical result could be explained by an ischemic damage of the SR with an increase in the open probability and/or conductance of the Ca^{2+}-release channels. Thus, ischemia may induce modifications of the SR-channel protein by different mechanisms, such as phosphorylation [29], changes in the redox state [15,30] or changes in the association of accessory proteins [31,32]. This effect of ischemia is analogous to that produced by hexachlorocyclohexane which induces an irreversible alteration of the RyR2 structure [33].

Accordingly, ischemia would change the channel structure increasing the rate constant of Ca^{2+} release despite a decrease in ryanodine binding. This interpretation is supported by our results because both alterations produced by ischemia, the increase in the rate of Ca^{2+} release and the SR damage, were prevented by preconditioning with tachycardia, suggesting that the increase in Ca^{2+} release was due to alteration of RyR2 channels. The decrease in the pentameric form of PLB, we observed following ischemia, implies an increase in its monomeric form, which in in vitro expressions systems appears to be a more effective Ca^{2+}-pump inhibitor than the PLB pentamer [34,35]. Consistent with these in vitro findings, overexpression of two PLB monomeric mutants (I40A and L37A) in transgenic mouse models has confirmed that the monomeric PLB mutants are superinhibitors of SERCA2 [36]. To the contrary, transgenic overexpression of another monomeric mutant (C41F), in the heart of PLB knockout mice leads to a reduced inhibition of cardiac relaxation compared to a similar level of expression of wild-type PLB, suggesting that pentameric assembly of PLB may enhance its inhibitory effect [37]. Thus, whether the monomeric or the pentameric form of PLB is a more effective inhibitor of the SR Ca-ATPase remains controversial. However, in the absence of a change in total PLB levels, the increase in SERCA2a protein levels could still underline a significant increase in Ca^{2+}-uptake activity.

The above results suggest that the preconditioning effect of tachycardia on the size of myocardial infarction, as we previously reported [6], is mediated at least partly through a protection of the integrity of the SR. This protection was associated with an early increase in SR Ca^{2+} release produced by the effect of tachycardia itself. Several reports [8–11,38–45] suggest that an increase in cytosolic Ca^{2+} is the triggering mechanism for preconditioning. Thus, a transient increase in intracellular [Ca^{2+}] by intracoronary infusion of Ca^{2+} would precondition canine [8,9] and rat [10] hearts through the PKC-signaling pathway [8,38]. In addition, the role of Ca^{2+} in ischemic preconditioning is supported by the abolition of the protection afforded by L-type Ca^{2+}-channel blockers in the isolated rat heart [10] and human atrial trabeculae [11]. These findings suggest that during the antecedent ischemic episode an increase in intracellular [Ca^{2+}], mediated by Ca^{2+} entry through voltage-dependent Ca^{2+} channels, triggers this protection. The observation [40] that in rat hearts three episodes of 5 min of ischemia followed by 5 min of reperfusion induce a rise in $[Ca^{2+}]_i$ agree with this proposal. However, the increase in intracellular [Ca^{2+}] was significantly smaller in the second and third ischemic episode, revealing a rapid adaptation of Ca^{2+} homeostasis to subsequent ischemic conditions. Moreover, the protective effects of diazoxide, a specific mitochondrial K^+_{ATP}-channel opener [41], on Ca^{2+} paradox [42] and on ischemia [43] are reverted when L-type Ca^{2+} channels are blocked with verapamil or nifedipine [42,43]. Therefore, it has been suggested that the protective effect of diazoxide could be due to a transient increase in cytosolic [Ca^{2+}] resulting from a decrease in mitochondrial Ca^{2+} uptake by the depolarizing effect of the drug [41]. Finally, Xu et al. [45] reported that a brief exposure of culture myocytes to Ca^{2+} prior to anoxia and reoxygenation (preconditioning by Ca^{2+}) reduces apoptosis by inhibiting the mitochondrial permeability transition and thereby the release of cytochrome *c* and the accumulation of Ca^{2+} in the mitochondria.

At variance with the above analysis, a different role for Ca^{2+} has been reported for the case of ischemic preconditioning. It has been proposed [21] that the antecedent ischemic episodes may produce a decrease in Ca^{2+} release by the SR that persists in time and prevents an increase in cytosolic [Ca^{2+}] during the prolonged subsequent ischemia (early ischemic preconditioning). According to Zucchi et al. [21] an antecedent ischemia of 3 min in the isolated rat heart induces, 5 min latter, a decrease in the density of ryanodine-binding sites and a decrease in the rate constant of Ca^{2+}-induced Ca^{2+} release that recovered after 240 min. Our results reveal that our preconditioning maneuver that is tachycardia induces an increase of SR Ca^{2+} release instead of a decrease as reported by Zucchi et al. [21] using ischemia as the preconditioning maneuver. However, both maneuvers induce the same de-

crease in the rate of Ca^{2+} release during a subsequent prolonged ischemia, that is a decrease in the rate of Ca^{2+} release. One possible explanation for the above discrepancy could be that the observed decrease in Ca^{2+} release measured 5 min after preconditioning with ischemia [21] may have followed a previous increase in cytosolic Ca^{2+} consequent to an inhibition of SERCA2 activity because of a reduced ATP phosphorylation potential [46]. As we mentioned above, Smith et al. [40] observed that the increase in Ca^{2+} release produced by a first brief episode of ischemia decreased in subsequent ischemic episodes of ischemia.

Our results do not allow us to ascertain how the preservation of the SR integrity by tachycardia is integrated in the cascade of reactions that lead to preconditioning. A transient early increase in cytosolic Ca^{2+} concentration by tachycardia may induce protection through the activation of PKC and activation of mitocondrial ATP-dependent K^+ channels [43]. In this regard, we reported previously [7] that the decrease in myocardial infarct size produced by tachycardia was reverted by blocking mitochondrial ATP-dependent K^+ channels. Accordingly, the preservation of SR as we observed in the present study would be a consequence of the above mechanism and a mediator in the preconditioning effect by preventing cytosolic Ca^{2+} overload. However, we cannot discard the possibility of a simultaneous and more active mechanism of protection of the SR integrity by a direct effect of tachycardia and its early release of Ca^{2+}. In this regard, we observed an increase in the level of the SR proteins RyR2 and SERCA2 as fast as 50 min after starting the episodes of tachycardia in dogs submitted only to tachycardia. We do not know whether these changes occur at the transcription, translation or post-translational levels. In rat hearts, an increase in mRNA and protein content of voltage-dependent K^+ channels at 30 and 90 min, respectively, after starting pacing has been reported [47]. This reveals early gene activation. On the other hand, Rowland et al. [48] found that ischemic preconditioning induces protein synthesis-dependent myocardial protection in the rat heart more than 1 h after preconditioning, an effect that was blocked by cycloheximide, a translator inhibitor but not by actinomycin, a transcriptor inhibitor.

In summary, our results suggest that short episodes of tachycardia prior to a prolonged ischemia protect the functional integrity of the SR, preventing cytosolic Ca^{2+} overload in myocardial cells. This effect could partly explain the preconditioning action of tachycardia.

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