

Factors Involved in the Susceptibility of Spontaneously Hypertensive Rats to Low K⁺-Induced Arrhythmias

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Abstract. Disorders of intracellular Ca²⁺ homeostasis and intercellular coupling are thought to be crucial in the initiation and maintenance of malignant arrhythmias. The aim of this study was to investigate possible arrhythmogenic factors in spontaneously hypertensive rats (SHR) as well as their susceptibility to low K⁺-related arrhythmias. The experiments were performed on isolated hearts of 13 weeks-old SHR and age-matched Wistar Kyoto rats (WKY). Equilibration of the heart by Langendorff perfusion with oxygenated, 37°C warm, standard Krebs solution at a constant pressure was followed by perfusion with low K⁺ solution for 60 min, unless sustained ventricular fibrillation occurred earlier. Electrocardiogram and epicardial monophasic action potentials (MAPs) were continuously monitored for incidence of arrhythmias and action potential changes. Myocardial tissue was taken for ultrastructural analysis and immunodetection of the main gap junction protein, connexin-43. The results showed that hypertrophic hearts of SHR exhibited prolongation of MAPs and a decrease in phosphorylation of connexin-43. Moreover, they were more prone to low K⁺-induced early after-depolarisations and ventricular premature beats as well as to connexin-43 and ultrastructural alterations than WKY rats. Consequently, the incidence of ventricular tachycardia (70% vs. 50%) and both transient (50% vs. 25%) and sustained (60% vs. 25%) ventricular fibrillation was higher in SHR than WKY rats. The results suggest that both prolongation of MAP and connexin-43 alterations are important arrhythmogenic factors facilitating arrhythmias in the setting of Ca²⁺ disorders due to hypokalaemia.

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Key words: SHR — Monophasic action potential — Connexin-43 — Hypokalaemia and arrhythmias

Introduction

Spontaneously hypertensive rats (SHR) are widely used as a model for the investigation of the myocardial alterations associated with hypertension, hypertrophy and heart failure. Ventricular hypertrophy under both experimental and clinical conditions is known to be associated with an increased susceptibility to arrhythmias (Swynghedauw et al. 1997; Zaugg et al. 1997). The mechanisms involved in this increased vulnerability are not fully understood. Recent studies suggest that it may be related to abnormal intracellular Ca^{2+} handling (Balke and Shorofsky 1998; Ebata et al. 1992) as well as to prolongation of repolarization (McIntosh et al. 1998). The increased level of cytoplasmic Ca^{2+} is thought to generate oscillatory electrical currents strong enough to cause recurrent action potentials and triggered automaticity (Hojo et al. 1992). Moreover, the excess of Ca^{2+} increases gap junctional resistance and inhibits intercellular coupling, thus facilitating the appearance of re-entry arrhythmias (Peters et al. 1997; Manoach and Tribulova 2001; Tribulova et al. 2002a).

Is the hypertrophic non-ischaemic cardiomyocyte more susceptible to changes in cytosolic Ca^{2+} ? The available data indicate that it is most likely so. Although the peak of the Ca^{2+} transient is unchanged, the duration of the transient is prolonged (McIntosh et al. 1998), thus it can be expected that this alteration can affect the stability of cellular ionic homeostasis. In consequence, any agent or condition that would increase cytoplasmic Ca^{2+} without affecting the buffering capacity of a normal cardiomyocyte will induce a profound disturbance in the Ca^{2+} homeostasis in the hypertrophic cardiomyocyte exhibiting fragile ionic equilibrium (Swynghedauw et al. 1997).

A rather large number of chronic or acute events are associated with abnormalities in Ca^{2+} handling, such as ischaemia, anoxia and hypokalaemia. Fibrosis and/or necrosis play an additional arrhythmogenic role by creating electrical heterogeneity within the cardiac tissue. Myocardial heterogeneity and electrical instability of structurally remodelled heart can be further enhanced by altered distribution of gap junctions as well as by disruption of intermyocyte coupling (Imanaga et al. 2002; Tribulova et al. 2002b). The consequences are nonuniform conduction and block of conduction associated with retrograde electrical activation “re-entry” and malignant arrhythmias (Peters et al. 1997).

Our previous results indicated that gap junction remodelling followed by acute impairment of the intercellular coupling due to increase in $[\text{Ca}^{2+}]_i$ facilitate occurrence of sustained atrial and/or ventricular fibrillation (Tribulova et al. 2001, 2002a, 2002b). We have found that the ability of the heart to ensure myocardial synchronization and electrical stability is decreased, if the number of gap junctions is reduced (Tribulova et al. 1999, 2002a; Okruhlicova et al. 2002).

The aim of this study was, therefore, to characterize the possible factors involved in the development of life-threatening arrhythmias in SHR hearts in the setting of experimental hypokalaemia.

Materials and Methods

The investigation conformed with the NIH Guide for the Care and Use of Laboratory Animals. Thirteen weeks-old, male, SHR ($n = 25$) and age-matched Wistar Kyoto rats (WKY) ($n = 28$) were used (Disease model, Co-operative Research Association, Kyoto). Under ether anaesthesia, the chest was open, the heart was quickly excised and the aorta was cannulated for Langendorff mode perfusion. The heart was equilibrated by 20 min perfusion with oxygenated (95% O₂ and 5% CO₂), 37°C warm Krebs solution containing (in mmol/l): NaCl 118, NaHCO₃ 25, KCl 2.9, MgSO₄ 1.2, CaCl₂ 1.8, KH₂PO₄ 1.3 and glucose 11.5 (pH 7.4) at a constant pressure (60 mm Hg). Bipolar ECG was continuously recorded *via* two stainless steel electrodes. Epicardial monophasic action potentials (MAPs) were recorded at two points *via* silver suction electrodes attached on the free wall of the right and the left ventricle, respectively. Coronary flow (CF) was measured by timed collection of coronary effluent. After 20 min of equilibration the hearts were perfused by K⁺-deficient (1.2 mmol/l) Krebs solution for a period of 60 min, unless sustained ventricular fibrillation (VF) (lasting 2 min) had occurred earlier. Sampling of tissue for analysis of connexin-43 and for ultrastructural examinations was performed at 20 min of equilibration period (WKY $n = 4$, SHR $n = 4$), in the early (5 ~ 7 min, WKY $n = 10$, SHR $n = 8$) and late (15 ~ 20 min, WKY $n = 11$, SHR $n = 8$) phase of low K⁺ perfusion, as well as at 2 min of sustained VF (WKY $n = 3$, SHR $n = 4$). The incidence of individual types of arrhythmias was estimated according to guidelines known as The Lambeth Conventions (Walker et al. 1988). Durations of MAPs (Franz et al. 1986) at 90% of repolarization were estimated manually (WKY $n = 7$, SHR $n = 7$).

Immunolabelling of the major gap junction protein, connexin-43 (Cx-43) was performed on 10 μm cryostat sections that were prepared from the middle part of the ventricles (control conditions $n = 25$ per group; low K⁺ perfusion $n = 25$ per group). Incubation with mouse antiCx-43 antibody was followed by incubation with secondary goat anti-mouse IgG antibody conjugated to FITC (Chemicon International). FITC signal was examined in a Carl Zeiss Jena fluorescence microscope.

For western blotting (WB), protein fractions obtained from the ventricles ($n = 3$ per group) were subjected to SDS polyacrylamide gels and electrophoretically transferred to Millipore membranes that were incubated with antiCx-43 antibody followed by incubation with alkaline phosphatase-conjugated secondary antibody. Two isoforms of Cx-43, phosphorylated (P1) and nonphosphorylated (P0) were detected. The occurrence of the P1 isoform was abolished by treatment with phosphatase and augmented by activation of protein kinase A. The amount of the P0 isoform was not affected by phosphatase treatment. The mean density of P1 and P0 isoforms of Cx-43 was measured using NIH Image and the data were plotted as

P1/P0 ratio for evaluation of the extent of phosphorylation of Cx-43 (Imanaga et al. 2002).

For examination of the ultrastructure, the heart was fixed by perfusion with buffered 2.5% glutaraldehyde. Epicardial, midmyocardial and endocardial tissue blocks (1 mm³) were excised, postfixated in OsO₄, dehydrated and embedded in Epon 812.1- μ m thick sections were examined in the light microscope and ultrathin sections from selected areas were examined in the electron microscope Tesla BS 500.

Statistical significance of differences between groups was determined by Student's *t*-test. Fisher's exact test was used for comparison of the incidence of ventricular tachycardia, transient and sustained ventricular fibrillation. The data were expressed as means \pm S.E.M., and the differences were considered as significant when $p < 0.05$.

Results

Compared to WKY rats, the SHR showed a significant increase in the systolic blood pressure (217 ± 9 vs. 138 ± 10 mm Hg, $p < 0.05$) and of the heart/body weight index (5.6 ± 0.02 vs. 3.5 ± 0.04 mg/g, $p < 0.05$) but not of body weight (283 ± 6 vs. 301 ± 8 g, $p > 0.05$). During stabilization of the perfused heart there were no significant differences in heart rate (265 ± 15 vs. 255 ± 10 beats/min) and in coronary flow (12 ± 1.0 vs. 12.5 ± 1.5 ml/min) between SHR and WKY rats. Perfusion with low K⁺ resulted in a decrease of heart rate (to 180 ± 18 and 195 ± 20 beats/min for

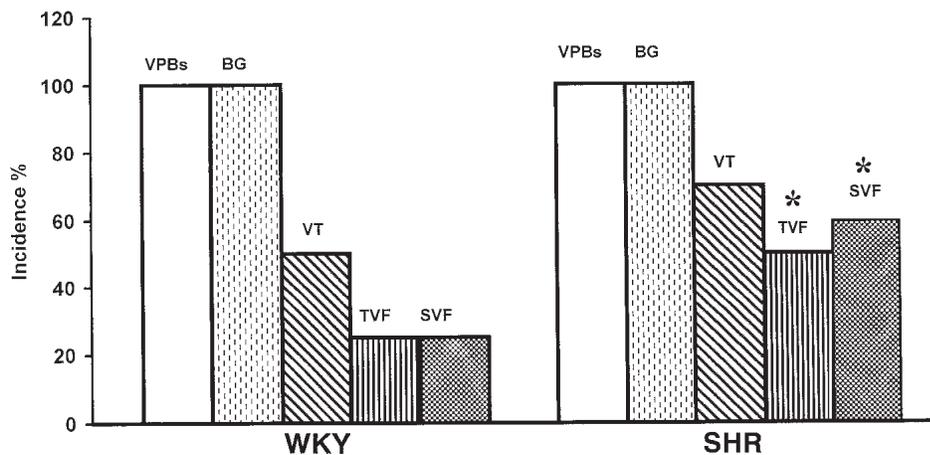


Figure 1. Susceptibility of WKY ($n = 12$) and SHR ($n = 20$) hearts to low K⁺-induced arrhythmias. VPBs, ventricular premature beats; BG, bigeminy; VT, ventricular tachycardia; TVF, transient and SVF, sustained (lasting more than 2 min) ventricular fibrillations. * significant difference ($p < 0.05$) between SHR and WKY rats.

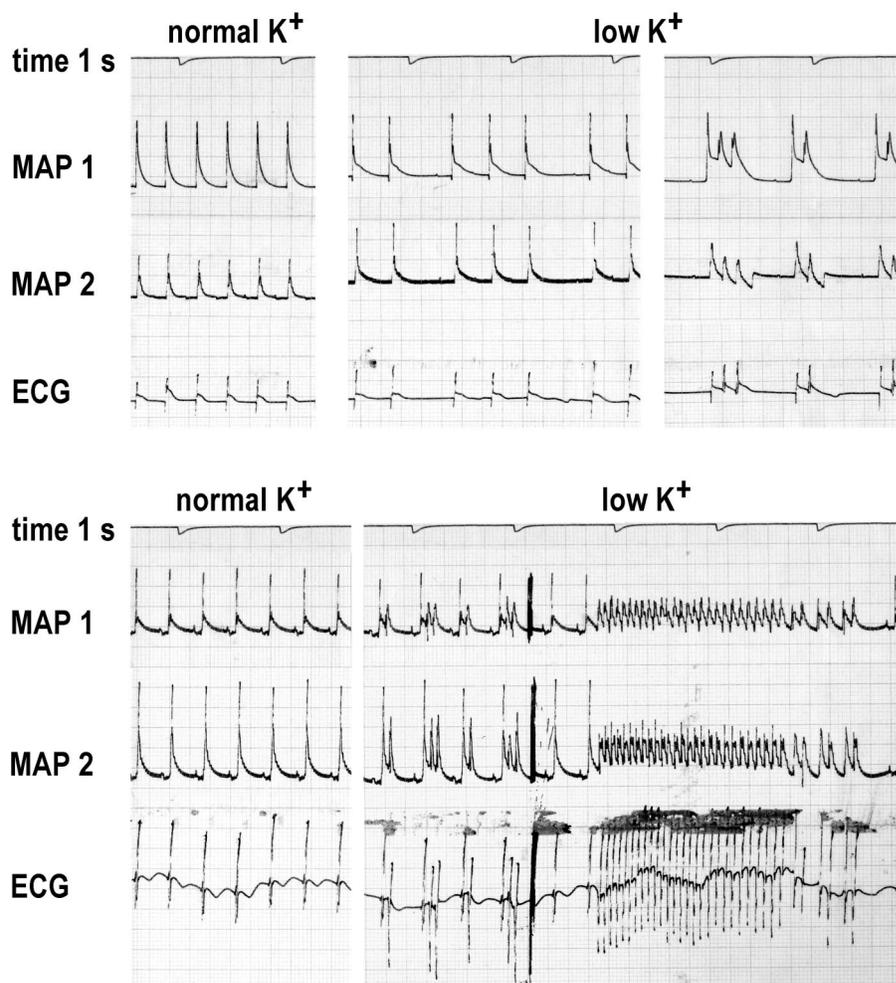
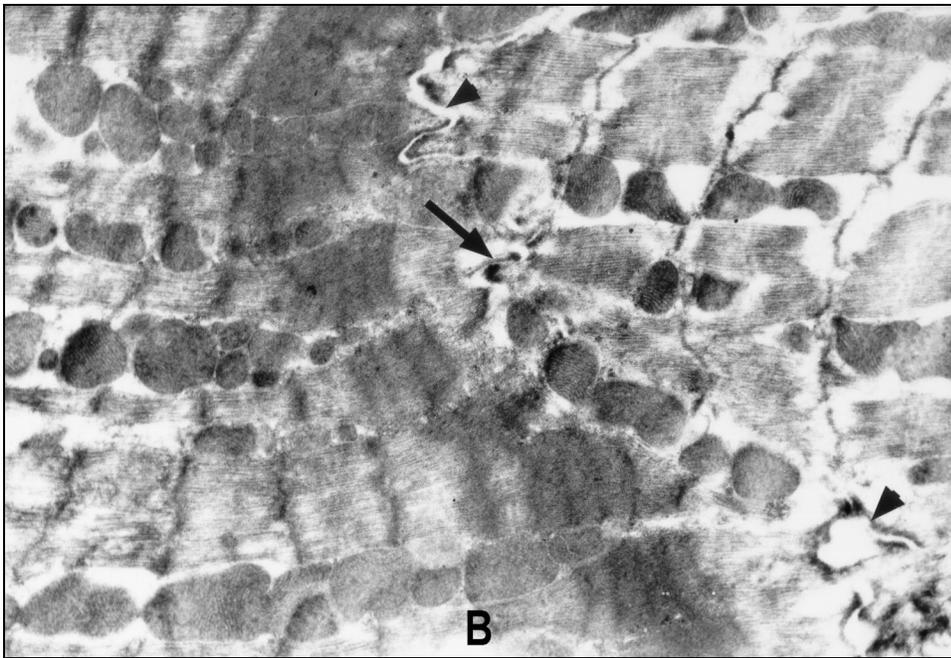
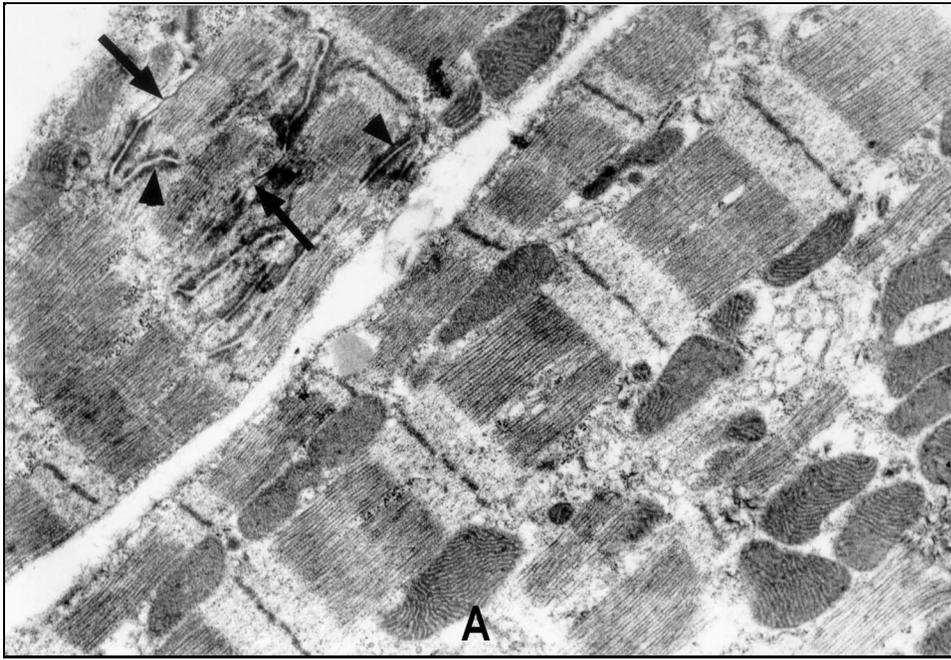


Figure 2. A typical original MAP1 (left ventricle), MAP2 (right ventricle) and ECG traces under normal conditions and after 10 min of low K^+ perfusion of WKY (upper panel) and after 5 min of low K^+ perfusion of SHR heart (bottom panel). Note the increased duration of MAPs in SHR already under normal conditions, as well as the decrease in heart rate and the increase in MAP duration in both groups due to low K^+ perfusion. The latter was associated with EADs (triggered in phase 2) in WKY (MAP1) and SHR (MAP2). VPBs and transient arrhythmias occurred earlier in SHR than in WKY rat hearts.

SHR and WKY rats, respectively) and of CF (to 6.9 ± 0.8 and 7.8 ± 1.2 and ml/min for SHR and WKY rats, respectively) that were significant upon 5 and 10 min of perfusion in SHR and WKY rats, respectively.



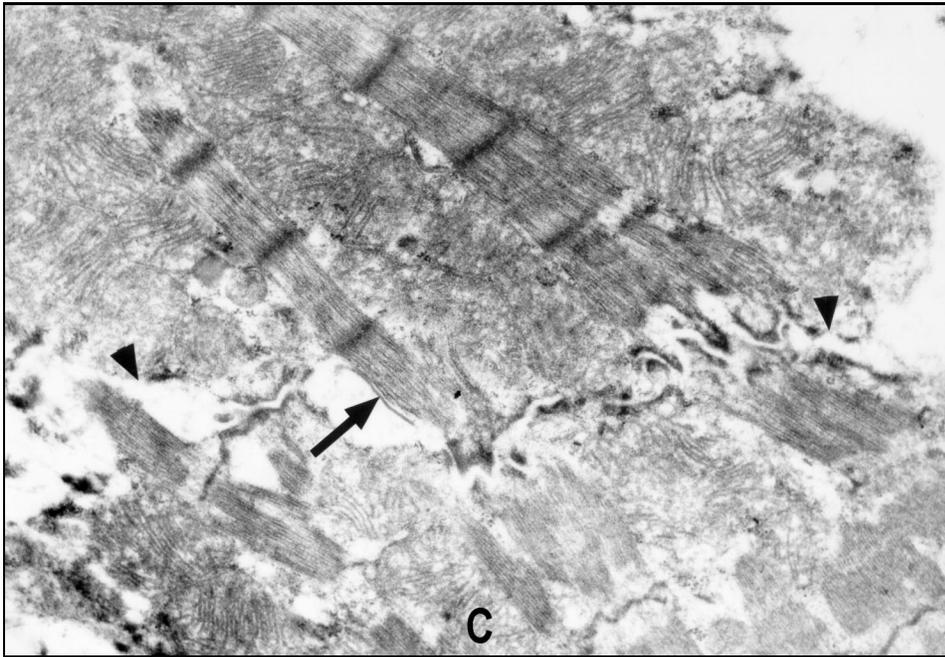
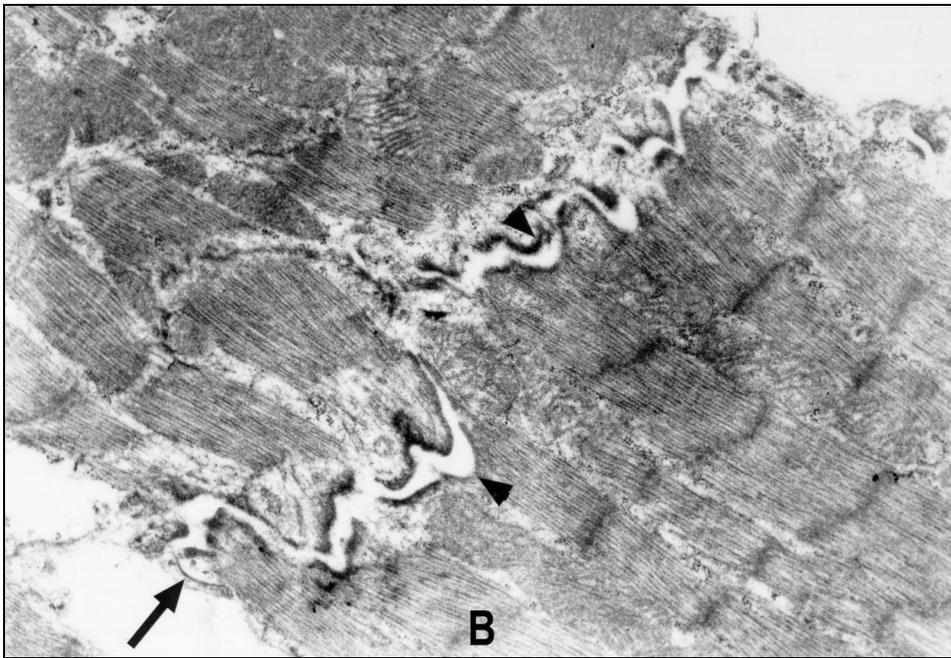
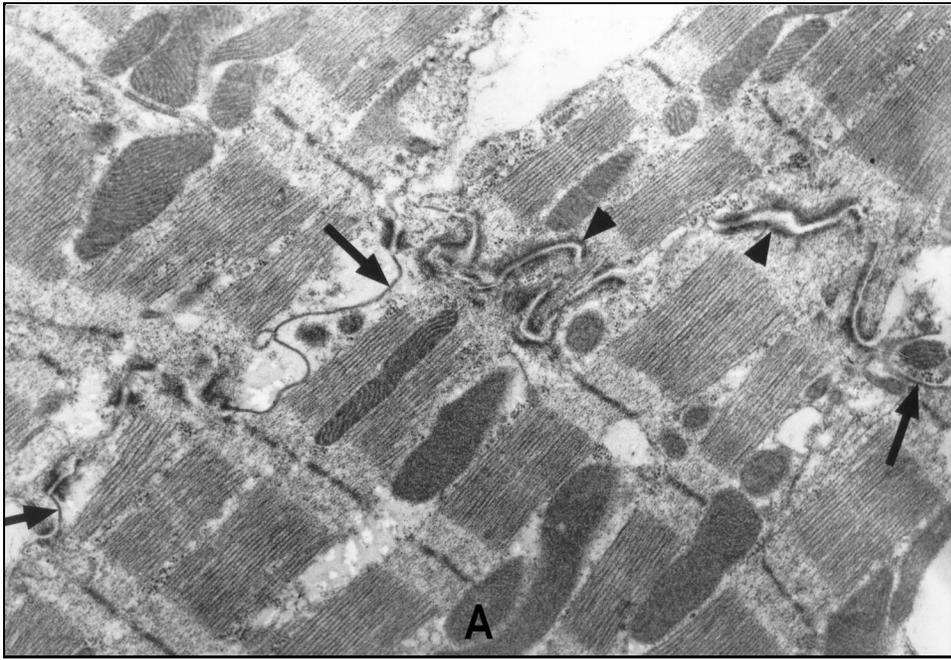


Figure 3. Electron micrographs of the WKY cardiomyocytes subjected to normal (A) and low K⁺ perfusion (B, C). Note end-to-end intercellular junctions (arrows – gap junctions, arrowhead – fascia adherens junctions) and subcellular alterations due to perfusion of the heart with low K⁺ solution, i.e., desynchronisation of contraction and overcontraction of sarcomeres as well as dehiscence of fascia adherens. These changes became more prominent after the occurrence of transient arrhythmias, resulting in loss of integrity of gap junctions (C). Magnification: 18,000.

The incidence of severe arrhythmias due to perfusion of the heart with low K⁺ solution was markedly and significantly higher in SHR hearts compared to WKY (Fig. 1), i.e., the incidence of ventricular tachycardia was 70% *vs.* 50%, the incidence of transient VF was 50% *vs.* 25%, and the incidence of sustained VF was 60% *vs.* 25%. There was a significant ($p < 0.001$) prolongation of MAP₉₀ in SHR hearts compared to WKY, 223.8 ± 14.5 *vs.* 115.1 ± 5.2 ms. Subjection of the heart to perfusion with a low K⁺ solution resulted in a significant ($p < 0.001$) lengthening of MAP duration in both groups to 221.5 ± 9.4 ms in WKY and to 259.2 ± 19.7 ms in SHR. In parallel with MAP prolongation there was an increase in the incidence of triggered electrical activity manifested by early after-depolarisations (EAD) in phase 2 of MAP that were linked with the occurrence of premature beats (Fig. 2). Importantly, triggered activity and ventricular premature beats (VPBs) already occurred within 3–5 min from the beginning of perfusion with low K⁺ solution in SHR, while they occurred with a delay (10–15 min) in WKY



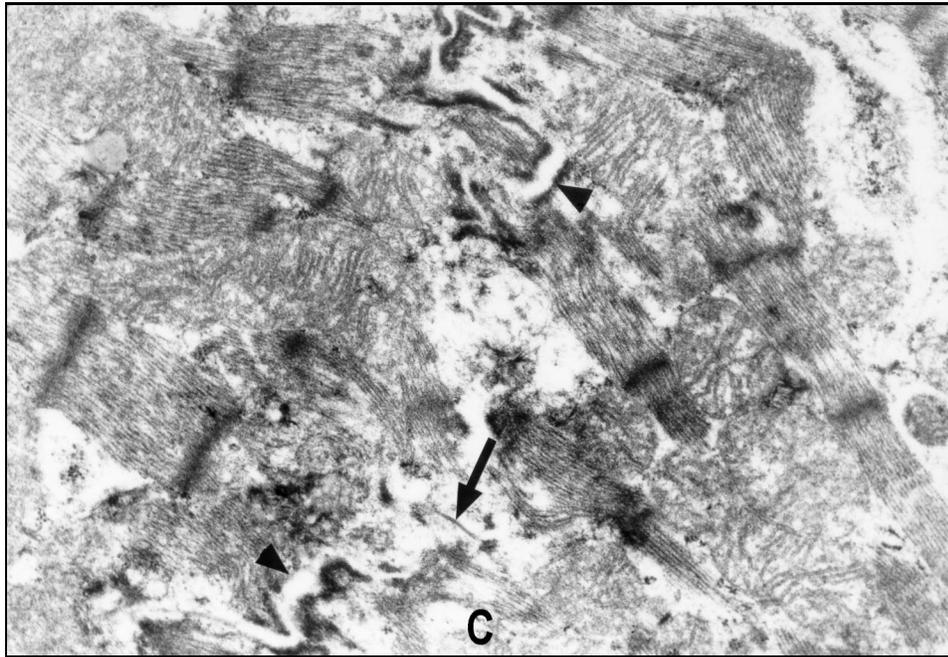


Figure 4. Cardiomyocytes from SHR heart (A) connected by both, end-to-end and side-to-side junctions (arrows – gap junctions, arrowheads – fascia adherens junctions). Markedly (B) and irreversibly (C) altered cardiomyocytes showing pronounced dissociation of fascia adherens junctions (B) and impairment of gap junctions, particularly in the tissue taken during transient arrhythmias (C). Note desynchronisation of contraction between adjacent cardiomyocytes. Magnification: 18,000.

hearts. Consequently, sustained VF appeared much earlier in hypertensive (after 10 min) than in normotensive (after 20 min) rat hearts.

Electron microscopic examination revealed conventional ultrastructure of cardiomyocytes with predominant end-to-end junctions in WKY (Fig. 3A). In contrast, hypertrophic cardiomyocytes of SHR possessed frequent side-to-side intercellular junctions (Fig. 4A). In both SHR and WKY rats, perfusion of the heart with low K^+ solution caused nonuniform reversible injury of cardiomyocytes, dehiscence of adhesive junctions and impairment of cell-to-cell gap junctional coupling. The latter can be inferred from the nonuniform patterns of sarcomeres indicating desynchronisation of contraction between neighbouring cardiomyocytes (Figs. 3B, 4B). The irregular contraction and over-contraction of sarcomeres strongly pointed on intracellular Ca^{2+} disorders as observed previously using $[Ca^{2+}]_i$ measurement (Tribulova et al. 2002a). All these changes occurred earlier and were more pronounced in the myocardium of SHR compared to WKY rats. Arrhythmias during low K^+ perfusion aggravated the injury, and under these conditions numerous car-

diomyocytes exhibited irreversible changes (Figs. 3C, 4C). There were no apparent differences in subcellular injury between epi-, mid- and endocardial regions.

Immunolabelling of Cx-43 did not show any differences in the overall density of myocardial gap junctions between SHR and WKY rats. However, in SHR hearts a higher number of lateral, side-to-side connections was apparent. In addition, micro-areas with loss of immunopositivity of Cx-43 were detected in hearts submitted to low K^+ (not shown) as observed previously (Tribulova et al. 2002a). Western blot analysis showed that there were no significant differences in the expression of Cx-43 between groups (data not shown). However, as exemplified in Fig. 5A the amount of the phosphorylated isoform of Cx-43 was much lower in SHR (line 5) compared to WKY (line 1), and it was further decreased by low K^+ perfusion (lines 6, 7, 8). Statistical analysis (Fig. 5B) showed that the differences were significant. A

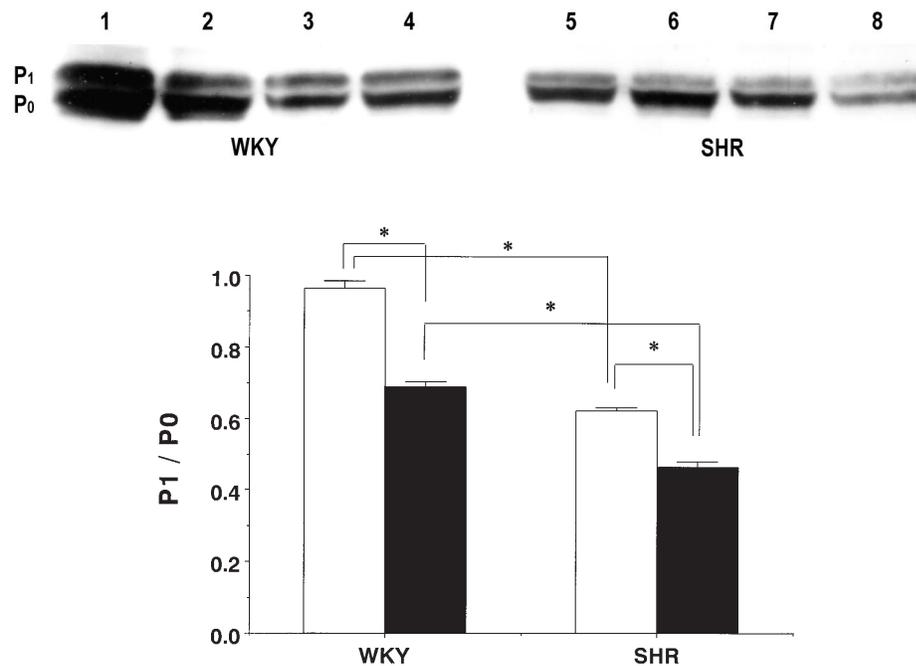


Figure 5. A. Western blots showing two forms of Cx-43 with different electrophoretic mobilities, phosphorylated (P1) and nonphosphorylated (P0), in WKY and SHR hearts. The P1 isoform is markedly suppressed in control SHR heart (line 5) compared to WKY (line 1). Low K^+ perfusion accompanied by transient arrhythmias resulted in a decrease in the P1 isoform in both WKY ($n = 3$, lines 2, 3, 4) and SHR ($n = 3$, lines 6, 7, 8) hearts. **B.** Western blotting data showing a significantly lower amount of the phosphorylated form of Cx-43 in SHR *vs.* WKY hearts under control conditions (white columns, $p < 0.001$), as well as a significant decrease in P1 induced by low K^+ perfusion with incidence of arrhythmias (black columns, $p < 0.001$) in both SHR and WKY rats.

decrease of the amount of the phosphorylated form of Cx-43 was detected also in WKY rat hearts exposed to K^+ deficient perfusion, as can be seen in Fig. 5A (lines 2, 3, 4) and Fig. 5B.

Discussion

The main finding of this study is that in SHR hearts exposed to experimental hypokalaemia, changes in myocardial repolarization (a prolongation of MAP duration) and in intercellular gap junctions (a decrease in Cx-43 phosphorylation) occurred in parallel with increased occurrence of arrhythmias. Moreover, we observed a close relationship between the prolongation of MAPs and the occurrence of EADs (linked with VPBs) in the whole heart preparation.

It has been reported that hypokalaemia-induced Ca^{2+} oscillations (Akita et al. 1998) probably account for the occurrence of VPBs due to EADs (January and Riddle 1989). Our data showing a significant increase in cytoplasmic free Ca^{2+} associated with premature beats (Tribulova et al. 2002a) strongly support this assumption. With increasing duration of low K^+ perfusion, the occurrence of VPBs was followed by transient arrhythmias (including VF). Likewise, hypokalaemia was shown in humans to be accompanied by QT-interval prolongation and the occurrence of ectopic activity followed by polymorphic tachycardia or "torsade de pointes" (Janko et al. 1992), which often degenerated into fibrillation. These arrhythmias may also occur during antiarrhythmic and other therapies that primarily prolong action potential duration (APD) and/or the QT-interval (Lazzara 1993). Hypokalaemia-related APD and Ca^{2+} disturbances are most likely due to suppression of K^+ currents (Janko et al. 1992) and Na,K-ATPase activity (Ziegelhoffer et al. 2000). Hypertrophic hearts of various etiologies may develop Ca^{2+} oscillations and triggered activity (Swynghedauw et al. 1997; Balke and Shorofsky 1998; McIntosh et al. 1998). Therefore, it can be expected that the hypertrophic heart of SHR, which exhibits prolongation of APD (Belichard et al. 1989) and/or MAP (shown in this study) will be prone to develop Ca^{2+} disturbances. Hypokalaemia further prolongs repolarization and contribute to deterioration of these changes. The higher vulnerability to Ca^{2+} overload and Ca^{2+} -related disorders of SHR hearts may result also from abnormal Ca^{2+} handling, i.e., from increased expression of Na^+/Ca^{2+} exchanger, suppression of sarcolemmal Na^+,K^+ -ATPase (Vrbjar et al. 2002) and Ca^{2+} -ATPase of the sarcoplasmic reticulum (Hojo et al. 1992; Qi et al. 1997). The increased susceptibility of SHR hearts to low K^+ -related Ca^{2+} disturbances may account not only for the earlier incidence of premature beats that initiate transient arrhythmias, but also for the significantly higher incidence of VF compared with WKY.

Triggered activity is known to precede ventricular tachycardia and fibrillation, whereby re-entry is considered as the underlying mechanism (Peters et al. 1997). Re-entry has been reported to be closely associated with both dispersion of myocardial repolarization and decreased intercellular gap junctional coupling (Manoach et al. 1986). Coordinated contraction of the heart depends on the rapid, orderly

propagation of action potentials *via* specialized channels clustered in gap junctions. Thus, electrical coupling *via* gap junction channels formed by the family of connexins is crucial for electrical synchronization. Turnover of channel proteins is high (half-life 1.5 hod) and their degradation involves both proteasome and lysosome pathways (Laing et al. 1997). Since Cx-43 is a phosphoprotein, changes in its phosphorylation state could modulate the degree of cell-to-cell coupling (Duthe et al. 2000; Imanaga et al. 2002).

Our results showing decreased phosphorylation of Cx-43 in SHR hearts as well as in the hearts perfused with K^+ deficient solution are in agreement with the above findings. At present we do not know why the ventricular tissue of SHR exhibits decreased phosphorylation status of Cx-43, as well as why perfusion with low K^+ further contributes to this decrease. However, we suggest that these changes are associated with functional channel alterations resulting in depressed communication (Cooklin et al. 1997; Duthe et al. 2000). Another key factor that inhibits gap junction channels and consequently deteriorates intercellular coupling is elevated cytoplasmic free Ca^{2+} (Delmar 2000; Manoach and Tribulova 2001; Imanaga et al. 2002). It is very likely, therefore, that Ca^{2+} overload that develops during low K^+ perfusion (Tribulova et al. 2002a) will impair cell-to-cell communication.

In conclusion, we suggest that prolongation of repolarisation and Cx-43 alterations that occurred in parallel can play a role in triggering of arrhythmias as well as in the higher vulnerability to ventricular fibrillation of the SHR heart. However, additional arrhythmogenic factors cannot be eliminated.

Acknowledgements. This work was partially supported by grant-in-aid of The vehicle racing commemorative foundation to I. I. and VEGA grants No. 2/3124/23, 2/2065/22 and 2/206422/22.

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Final version accepted: March 26, 2003