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Dispersion of Cell-To-Cell Uncoupling Precedes Low K^+-Induced Ventricular Fibrillation

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Summary

We hypothesize that hypokalemia-related electrolyte imbalance linked with abnormal elevation of intracellular free Ca^{2+} concentration can cause metabolic disturbances and subcellular alterations resulting in intercellular uncoupling, which favor the occurrence of malignant arrhythmias. Langendorff-perfused guinea pig heart (n = 44) was subjected to a standard Tyrode solution (2.8 mmol/l K^+) followed by a K^+-deficient solution (1.4 mmol/l K^+). Bipolar ECG of the left atria and ventricle was continuously monitored and the incidence of ventricular fibrillation was evaluated. Myocardial tissue sampling was performed during stabilization, hypokalemia and at the onset of fibrillation. Enzyme activities of succinic dehydrogenase, glycogen phosphorylase and 5-nucleotidase were determined using *in situ* catalytic histochemistry. The main gap junction protein, connexin-43, was labeled using mouse monoclonal antibody and FITC conjugated goat antimouse antibody. Ultrastructure was examined by transmission electron microscopy. The free Ca^{2+} concentration was measured by the indo-1 method in ventricular cell cultures exposed to a K^+-free medium. The results showed that sustained ventricular fibrillation appeared within 15-30 min of low K^+ perfusion. This was preceded by ectopic activity, episodes of bigeminy and tachycardia. Hypokalemia induced moderate reversible and sporadically irreversible subcellular alterations of cardiomyocytes and impairment of intercellular junctions, which were heterogeneously distributed throughout myocardium. Patchy areas with decreased enzyme activities and diminished immunoreactivity of connexin-43 were found. Furthermore, lack of external K^+ was accompanied by an increase of intracellular Ca^{2+}. The prevention of Ca^{2+} overload by either 1 mmol/l Ni^{2+} (Na^+/Ca^{2+} inhibitor), 2.5 μmol/l verapamil, 10 μmol/l d-sotalol or 10 μmol/l tedisamil was associated with the protection against fibrillation. The results indicate that hypokalemia induces Ca^{2+} overload injury and disturbances in intercellular coupling. Dispersion of these changes throughout the myocardium may serve as the basis for microreentry circuits and thus favor fibrillation occurrence.

Key words
Hypokalemia • Ventricular fibrillation • Ca^{2+} • Connexin-43 • Intercellular junctions

Introduction

Potassium is known to be one of the possible chemical mediators of potentially life threatening arrhythmias, whereby both an increase and a decrease of external K^+ might be arrhythmogenic (Curtis *et al.* 1993). Hyperkalemia in experimental as well as clinical studies is linked particularly with ischemic conditions and ischemia-induced ventricular fibrillation (Curtis *et al.* 1993), whereas hypokalemia is particularly associated with the incidence of torsade de pointes during diuretic and other therapies (Steiness and Olesen 1976). Moreover, hypokalemia is frequently associated not only with cardiovascular but also with gastrointestinal and urogenital disturbances (Janko *et al.* 1992). Both, transient hyperkalemia or hypokalemia can be induced by increased catecholamines, stress or exercise (O'Neill *et al.* 1993, Seck *et al.* 1996). These conditions can play a role in sudden cardiac death in otherwise healthy subjects. Despite the data indicating that hypokalemia decreases myocardial electrical stability by altering cardiac excitability, by increasing the membrane potential, duration of the action potential and effective refractory period and by decreasing the conduction velocity (Akita *et al.* 1998), the whole spectrum of low K^+-induced alterations involved in the initiation of ventricular fibrillation is still uncertain.

Our previous studies dealing with mechanisms involved in the appearance of transient versus sustained ventricular fibrillations (Manoach *et al.* 1987) as well as our recent examination of electrically (Tribulová *et al.* 1998) and/or ischemia-induced (Ravingerová *et al.* 1995) cardiac fibrillations have indicated that the viability of cardiomyocytes and impairment in intercellular junctions and coupling can be critical in the process of initiation and persistence of asynchronous activity, which can degenerate into fibrillation. We reported the inhibition of gap junctional communication and uncoupling, most likely induced by excess of Ca^{2+} during hypoxia (Manoach *et al.* 1996) or elevated external Ca^{2+} concentration in ventricular muscle strips (Uchiyama *et al.* 1995) and cultured ventricular myocytes (Manoach *et al.* 1997). This was accompanied by asynchronous contractions which were returned to normal by drugs normalizing intracellular Ca^{2+} concentrations (Manoach *et al.* 1997). Gap junctional uncoupling was shown to be associated with discontinuous propagation and nonuniform anisotropy in small circuits, which can initiate reentry (Spach and Heidlage 1995).

Since the mechanism by which hypokalemia affects cardiac metabolism, structure and function to elicit life threatening arrhythmias is still unclear, the aim of this study was to examine some of these parameters, focusing particularly on alterations in intermyocyte junctions and coupling.

Material and Methods

The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health, Publication No 85-23, revised 1996.

The experiments were conducted on adult guinea pig hearts (n=44) of both sexes. The animals were sacrificed by stunning followed by carotid exsanguination and the aorta of the excised heart was immediately cannulated for perfusion with 37 °C warm Tyrode solution (in mmol/l: 136.9 NaCl, 2.8 KCl, 1.8 $CaCl_2$, 1.0 $MgCl_2$, 11.9 $NaHCO_3$, 0.4 NaH_2PO_4, 5.5 glucose) oxygenated by 95 % O_2 and 5 % CO_2 at the pressure of 70 mm Hg. After 15 min stabilization with standard solution (2.8 mmol/l K^+), the heart was perfused with a low K^+ solution (1.4 mmol/l). Bipolar epicardial electrocardiograms from the left atrium and ventricle were continuously monitored and the incidence of arrhythmias was evaluated.

The antifibrillatory efficacy of Ca^{2+} channel blockers, verapamil (2.5 μmol/l) and diltiazem (4 μmol/l), Na^+/Ca^{2+} inhibitor Ni^{2+} (1 mmol/l) and class III drugs, d-sotalol (10 μmol/l) and tedisamil (10 μmol/l), was examined (n = 4-6 hearts for each drug).

Cytosolic $[Ca^{2+}]i$ was estimated by indo-1 method in 3- to 6-day-old cultured rat ventricular cardiomyocytes bathed in normal and K^+-depleted Tyrode solution as described previously (Manoach *et al.* 1997).

Left ventricular tissue was collected during stabilization, low K^+ perfusion and at the onset of ventricular fibrillation. For *in situ* demonstration of succinic dehydrogenase (1.3.99.1), glycogen phosphorylase (2.4.1.18) and 5-nucleotidase (3.1.3.5) enzyme activities and for immunodetection of gap junction protein connexin-43, the left ventricle was immediately frozen in liquid nitrogen and cut into 10 μm thick cryostat sections. The immunolabeling of connexin-

43 was performed using monoclonal mouse anticonnexin-43 Ab and FITC goat antimouse IgG (Zymed Laboratories Inc.). Histochemical and immunostaining reactions were examined under light and/or fluorescence microscopes, respectively (Carl Zeiss Jena).

For transmission electron microscopic examinations, small tissue blocks of the left ventricle were fixed with 2.5 % glutaraldehyde in 0.1 mol/l sodium cacodylate, postfixed in 1 % osmium tetroxide, dehydrated in ethanol, infiltrated by propylene oxide and embedded in Epon 812. The ultrathin sections were stained with uranyl acetate and lead phosphate and examined in an electron microscope Tesla 500.

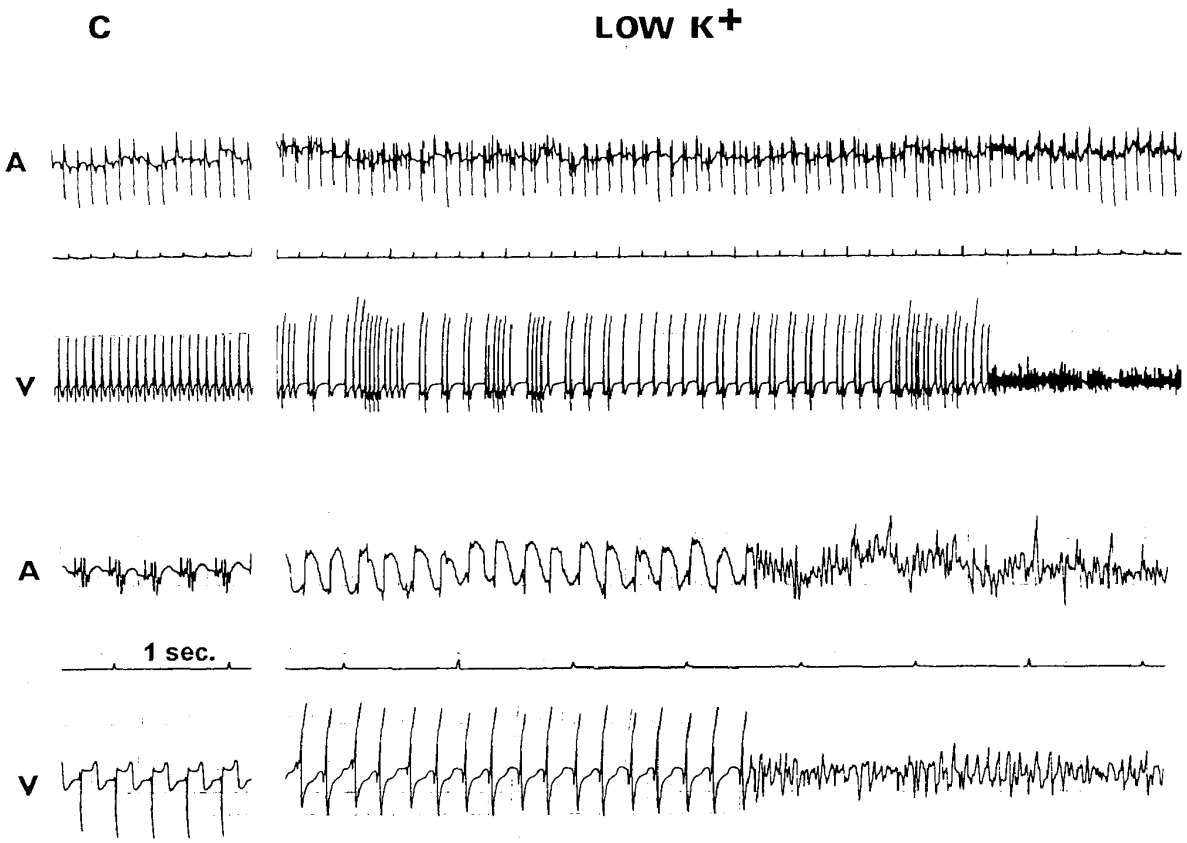

Fig. 1. *ECG records from two experiments on isolated guinea pig hearts during stabilization perfusion with standard Tyrode solution (C) and during 20-30 min with a K^+-deficient solution. Salves of bigeminy and tachyarrhythmias or changes in the R vector preceded the occurrence of ventricular fibrillation. A – left atrium; V – left ventricle, time in seconds.*

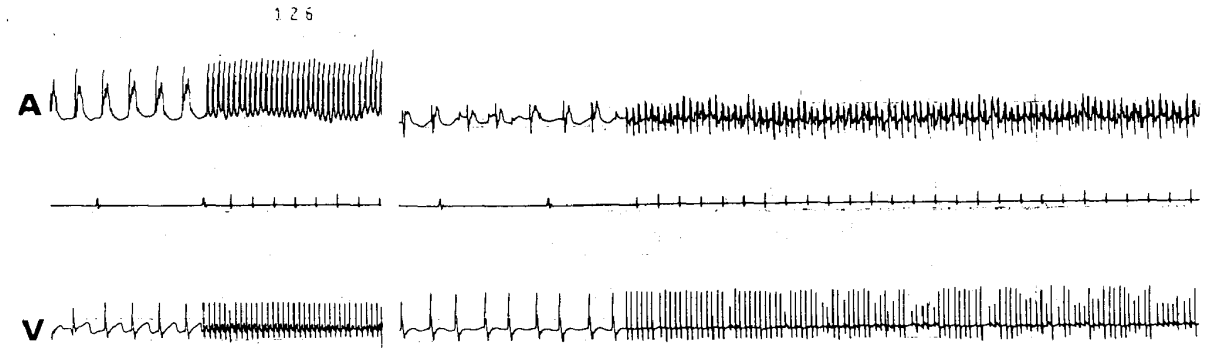

Fig. 2. *Guinea pig heart subjected to 30 min perfusion with a K^+-deficient Tyrode solution containing 1 mmol/l Ni^{2+} to prevent ventricular fibrillation. A – left atrium, V– left ventricle, time in seconds.*

Results

Perfusion of the isolated guinea pig heart with a K^+-deficient Tyrode solution lasting 15-30 min induced 100 % incidence of sustained (lasting more than 2 min) ventricular fibrillation. The continual recordings of ventricular bipolar electrocardiograms during hypokalemia exhibited changes in R and T configuration, bigeminy, ectopic activity followed by clear changes in the R vector and sudden ventricular tachycardia, which preceded sustained ventricular fibrillation (Fig. 1).

Administration of verapamil and diltiazem (not shown) or Ni^{2+} decreased the incidence of transient arrhythmias and fully prevented the occurrence of sustained ventricular fibrillation (Fig. 2), whereas both tedisamil and d-sotalol (not shown) reversed ventricular fibrillation into a sinus rhythm in all examined hearts (Fig. 3).

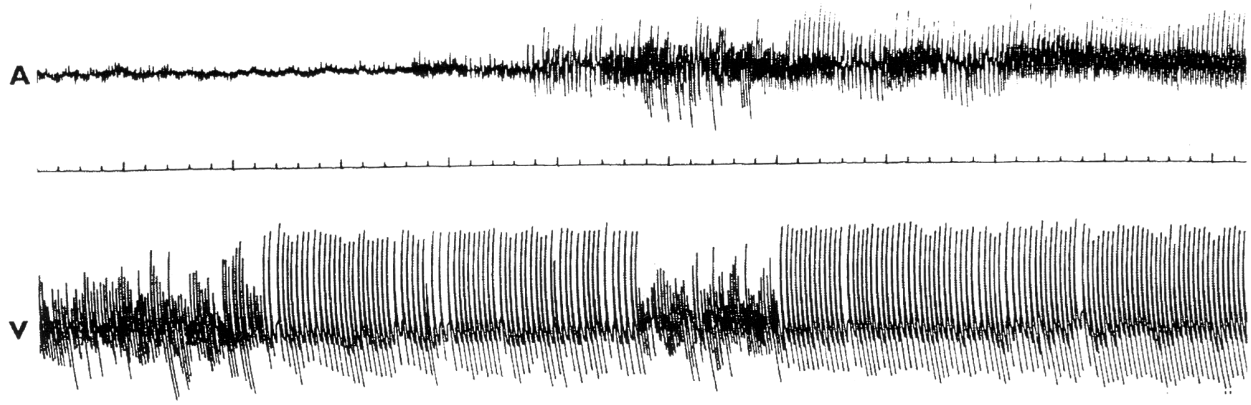

Fig. 3. *Administration of 10 μmol/l tedisamil during low K^+-induced ventricular fibrillation caused clear ventricular defibrillation. A – left atrium, V – left ventricle, time in seconds.*

During stabilized perfusion the myocardium did not exhibit any alterations in the histochemically determined enzyme activities. The product of histochemical reaction was uniformly distributed within the myocardium. Low K^+ perfusion induced patchy areas with abolished histochemical reactions, reflecting markedly decreased enzyme activities. These areas were present in the vicinity of cardiomyocytes with a normal, uniform reaction (Figs 4A, 4B and 4C). Moreover, irregular patterns of the succinic dehydrogenase reaction at the interface of moderately and severely affected cardiomyocytes, were frequently found. All these changes were more pronounced in the fibrillating myocardium.

Immunodetection of connexin-43 in control perfused hearts, revealed numerous gap junctions regularly distributed at the site of intercalated discs (Fig. 5A). The K^+-deficient perfusion altered the distribution of connexin-43 as well as patchy areas with a diminished or absent immunoreaction were observed (Figs 5B and 5C). This feature was more often observed in the myocardium from fibrillating hearts (Fig. 5D).

Ultrastructural evaluation showed that in comparison to normal subcellular architecture of the cardiomyocytes and preserved intermyocyte junctions (Fig. 6A), the myocardial tissue from the heart subjected to K^+-deficient perfusion was characterized by reversibly altered cardiomyocytes as well as by irreversibly injured cardiomyocytes (Fig. 6B). Nonuniformly affected cardiomyocytes were heterogeneously distributed throughout the myocardium. Severely damaged cardiomyocytes were edematous, with apparently injured mitochondria and impaired integrity of intermyocyte junctions at the fascia adherens and gap junctions. Less affected cardiomyocytes exhibited mild edema and moderate mitochondrial alterations as well as a mild dissociation of adhesive junctions (fascia adherens) and no visible changes in gap junctions. However, the neighboring cardiomyocytes showed nonuniform contractions of myofibers (Fig. 7A), indicating disturbances in cell-to-cell synchronization. Moreover, the occurrence of hypercontracted myofibers and even contraction bands, especially in irreversibly altered cardiomyocytes, indicated Ca^{2+} overload injury. All the described changes were much more apparent in the fibrillating myocardium (Fig. 7B).

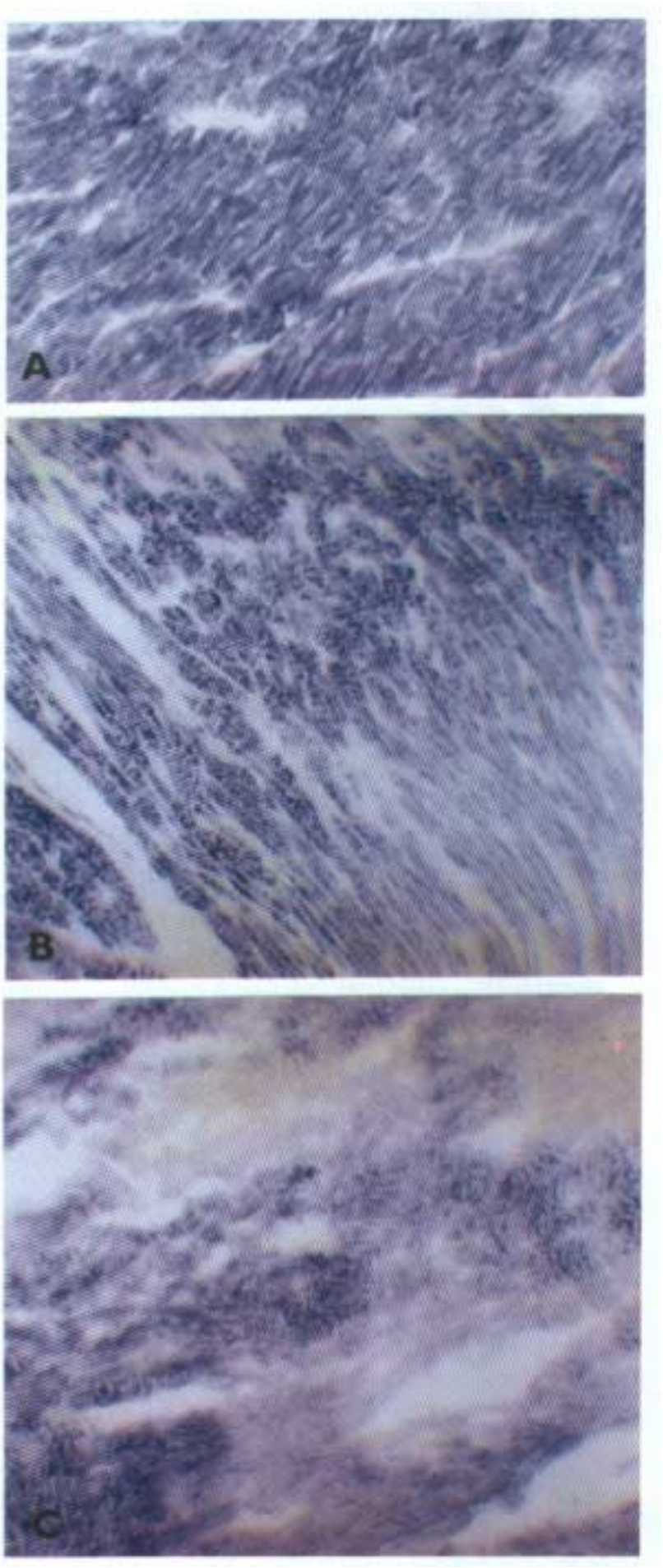

Fig. 4. *Histochemical demonstra-tion of succinic dehydrogenase activity in the myocardium during perfusion with Tyrode solution (A). 25 min of K^+-deficient perfusion induced nonhomo-geneity of the histochemical reaction throughout the myocardium and areas with decreased activity and irregular staining, reflecting hyper-contraction of myofibers, were observed (B). Microareas with abolished reaction and absent succinic dehydrogenase activity were more pronounced in the fibrillating myocardium (C). Magnification 80x.*

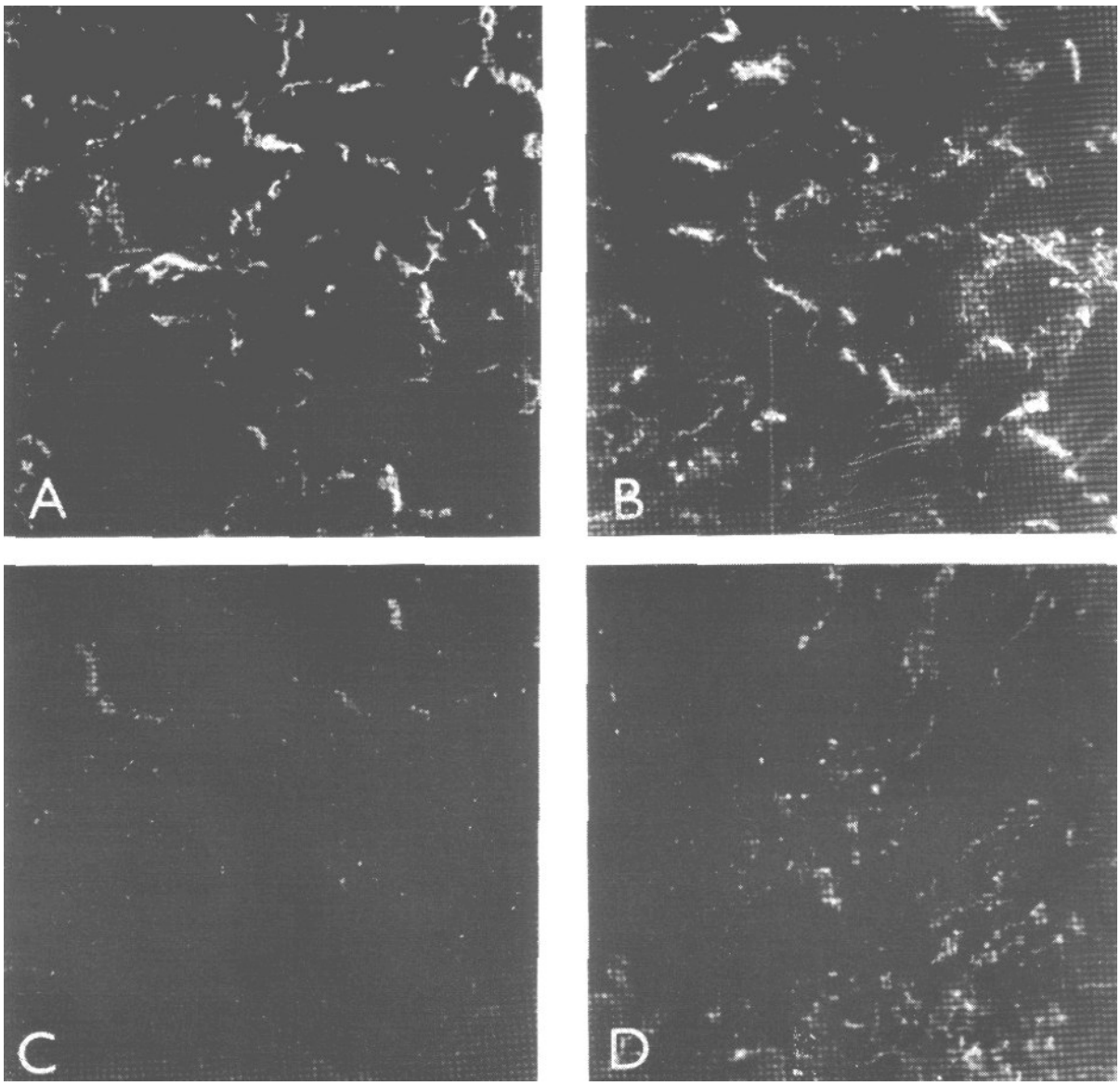

Fig. 5. *Immunolabeling of connexin-43 and normal pattern of gap junctions in the left ventricle during stabilization perfusion (A). After 15-30 min of low K^+ perfusion disorganized gap junctions and punctate pattern of staining were observed (B). Moreover, small areas with a decreased number of immunolabeled gap junctions were found (C). Punctate pattern of staining and areas with diminished immunoreaction were more pronounced in fibrillating left ventricle (D). Magnification 80x.*

Hypokalemia was associated with elevation of cytoplasmic free calcium. In 3- to 6-day-old cultured ventricular cardiomyocytes subjected to the K^+-free Tyrode solution, intracellular Ca^{2+} concentrations were significantly increased from 100 to 340 nmol/l (Fig. 8).

Discussion

Our results have shown that isolated guinea pig hearts, subjected to low K^+ perfusion for a relatively short period, exhibit ectopic activity, episodes of premature beats, bigeminy and tachycardia followed by sustained ventricular fibrillation. The hypokalemia-induced electrolyte disturbances are accompanied by heterogeneously distributed histochemical, ultrastructural and connexin-43 gap junction-related myocardial alterations.

In human and experimental models, hypokalemia is linked particularly with polymorphic tachycardia or torsade de pointes, which often degenerate

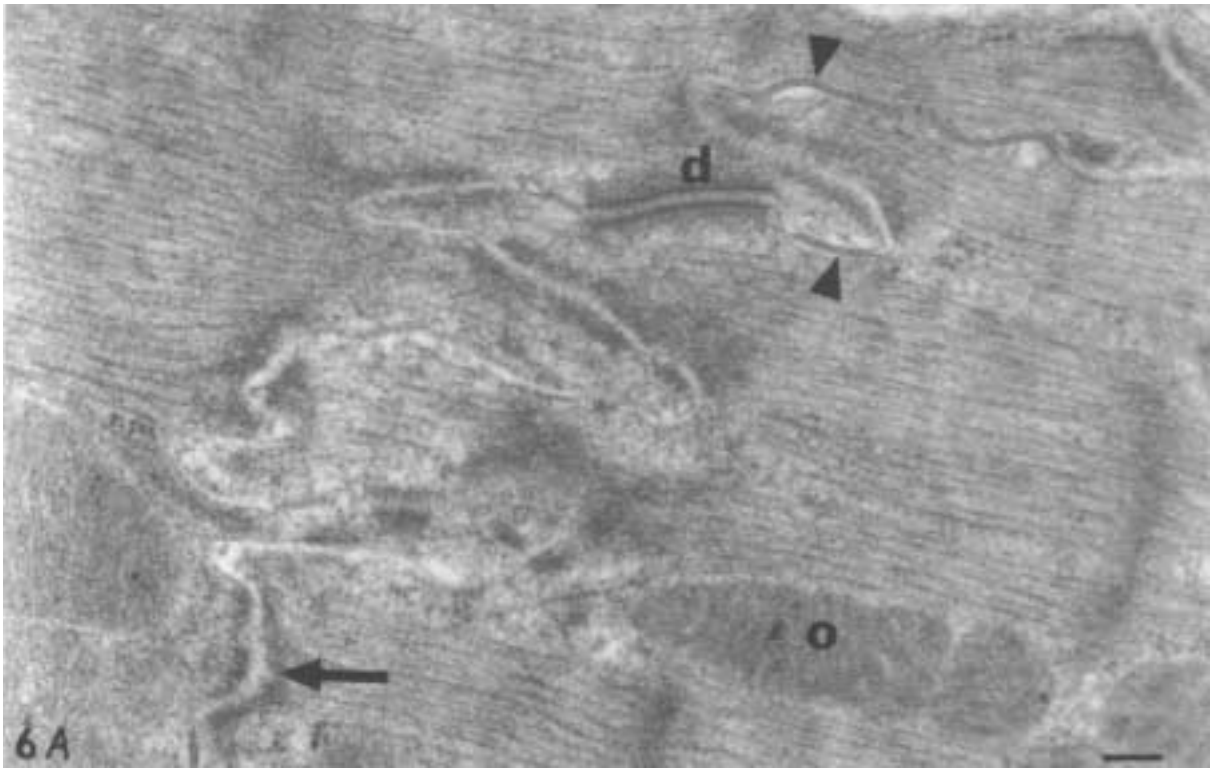

Fig 6A. *Ultrastructure of the cardiomyocyte from control left ventricle showing normal appearance of three types of intermyocyte junctions. Fascia adherens (arrow) and desmosome (d) are responsible for mechanical coupling, i.e. developed force transduction. Gap junctions (arrowheads) are particularly responsible for electrical coupling, i.e. action potential propagation and synchronization, but also for metabolic coupling, i.e. intermyocyte signal transduction. o – mitochondria. Bar represents 1 µm.*

into fibrillation. These potentially fatal arrhythmias were closely associated with early or late afterdepolarizations due to prolongation of action potential duration (APD) and elevation of free Ca^{2+} concentration that also occurred during antiarrhythmic therapy, which primarily prolongs APD and refractoriness (Lazzara 1993).

Besides suppressing Na,K-ATPase activity (Knochel 1987), hypokalemia decreased the activity of sarcolemmal 5-nucleotidase indicating disturbances in purine nucleotide metabolism. This coincides with a decrease of ATP concentrations and impaired respiratory activity of mitochondria (Shapiro *et al.* 1998), which was demonstrated histochemically as decreased mitochondrial succinic dehydrogenase activity (Fig. 4) and by diminished cytoplasmic glycogen phosphorylase activity. These disturbances can contribute to a further deterioration of low K^+-induced changes, such as accumulation of cytoplasmic free calcium. This increase of Ca^{2+} influx resulting most likely from hypokalemia-induced prolongation of APD can be enhanced by dysfunction of the sodium pump (Na,K-ATPase) with consequent reverse mode of the Na^+/Ca^{2+} exchanger.

Excess of Ca^{2+} and Ca^{2+} overload may favor abnormal automaticity, triggered activity and induce aftercontractions (Helfant 1986). This can explain the occurrence of various arrhythmias recorded before the occurrence of fibrillation (Fig. 1). Both increased Ca^{2+} in cultured rat cardiomyocytes exposed to a K^+-free solution (Fig. 8) and hypercontractions observed in guinea pig hearts perfused with low K^+ indicate disturbances in Ca^{2+} homeostasis. These findings are in accordance with this explanation.

Taking into consideration that not only chronic but also acute hypokalemia (Shapiro *et al.* 1998) increase cytoplasmic Ca^{2+}, we suggest that the abnormal elevation of Ca^{2+} and/or Ca^{2+} overload with its deleterious effects particularly on intermyocyte coupling is the main factor involved in hypokalemia-induced arrhythmias and fibrillation. This assumption is supported by our findings that the examined compounds, which either prevented or

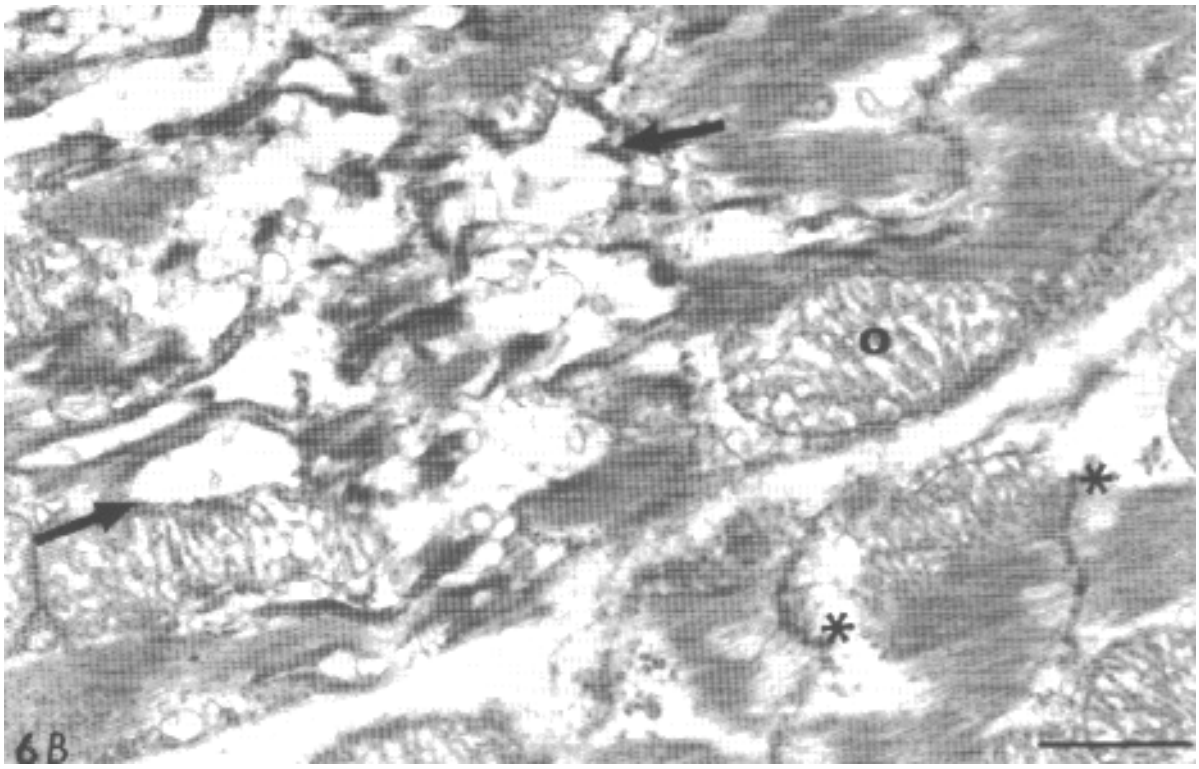

Fig. 6B. *Perfusion with low K^+ for 20-30 min induced heterogeneity in the subcellular alterations. Severely injured cardiomyocyte with edematous mitochondria (o) and prominent dehiscence of fascia adherens junctions (arrows). Gap junctions are not visible. Nonuniform pattern of sarcomeres (asterisks) indicate disorganization of contraction. Bar represents 1 μm.*

attenuated Ca^{2+} overload, exhibited clear antifibrillating/ defibrillating effects. Thus, not only Ca^{2+} entry blockers (verapamil and Ni^{2+}) but also class III antiarrhythmic drugs, d-sotalol and tedisamil, which are able to decrease elevated Ca^{2+} concentration (Manoach *et al.* 1996, Tribulová *et al.* 1999a) exhibited beneficial effects against fatal arrhythmia.

It was established that high Ca^{2+} inhibits gap junctional channels and downregulates intermyocyte communication (DeMello 1986), which was manifested by decreased conduction velocity (Guerrero *et al.* 1997, Thomas *et al.* 1998) and particularly by myocardial electrical uncoupling (DeMello 1986, Uchiyama *et al.* 1995, Manoach *et al.* 1996). Cell-to-cell uncoupling and loss of electrical and metabolic synchronization provided by gap junction channels can cause nonhomogeneity in APD (Joyner 1982) and probably abolish the physiological heterogeneity of APD.

Indeed, hypokalemia-induced electrolyte disturbances resulting in the accumulation of Ca^{2+} and metabolic alterations were accompanied by changes in immunodetection of the major gap junction protein connexin-43. Similarly to the heterogeneously decreased enzyme activities, disseminated microareas with decreased or abolished immunoreaction of connexin-43 were observed (Fig. 5B). The latter clearly indicate local impairment of gap junctions (Peters *et al.* 1995, Peters 1996) suggesting disturbances in electrical coupling and most likely intermyocyte uncoupling in these areas. We have found similar alterations in the myocardial tissue during burst pacing-induced sustained atrial fibrillation (Tribulová *et al.* 1999b).

The alterations related to connexin-43 were detected for the first time in acute models resulting in cardiac fibrillation and they coincide in some way with alterations in connexin-43 or -40 (van der Velden *et al.* 1996) detected in chronic experiments or in the human diseased heart prone to arrhytmias and fibrillation (Peters 1996, Peters *et al.* 1997). Both chronic and acute

Fig. 7A. *Reversibly injured two cardiomyocytes connected with slightly altered intermyocyte junctions, however, exhibiting desynchronization of contraction, i.e. contracted sarcomeres in one and relaxed in another cardiomyocyte. D – desmosome, arrow – fascia adherens, arrowhead – gap junction, o – mitochondria, asterisks – nonuniform pattern of sarcomeres. Bar represents 1 μm.*

alterations characterized by nonuniform gap junctional distribution may underlay abnormal electrical conduction (Saffitz *et al.* 1992, Severs 1994). Lost or decreased intensity of immunofluorescence can be attributed to degradation of gap junction proteins as well as to their conformational changes and masking of connexin-43 epitops (Doble *et al.* 1996, Laing *et al.* 1998). Accordingly, degradation and permanent loss of immunoreactivity can be associated particularly with irreversibly damaged cardiomyocytes. These were surrounded by less affected cardiomyocytes probably with reversible alterations of gap junction channels and a transient form of intermyocyte uncoupling. We suggest that the local and dispersed character of these myocardial changes might provide the substrate (Tribulová *et al.* 1998) for multiplying the circuits of reentry similarly as was suggested for border infarct zones (Peters *et al.* 1997).

Ultrastructural evaluation demonstrated a heterogeneous population of cardiomyocytes possessing in the majority of cases reversible but also irreversible alterations. The occurrence of hypercontraction of myofibers and sporadically even contraction bands indicated Ca^{2+} overload-related injury. Moreover, cardiomyocytes with more or less impaired intercellular junctions were found. Nonuniform patterns of sarcomeres in neighboring cardiomyocytes have indicated desynchronization of contraction, most likely resulting from intermyocyte uncoupling. In accordance with the observed histochemical and connexin-43 changes, similar features of subcellular alterations were found in the rat heart prone to ischemia and/or reperfusion-related arrhythmias (Ravingerová *et al.* 1995, Tribulová *et al.* 1993) or in the guinea pig heart during periods of burst pacing resulting in sustained atrial fibrillation (Tribulová *et al.* 1999b). They point to a close relationship between heterogeneously injured, but still viable cardiomyocytes, and the occurrence of fibrillations.

Thus, despite species-related differences and multiplicity of potentially contributing factors involved in the arrhythmogenesis (Janse *et al.* 1998), disturbances in Ca^{2+} homeostasis and high Ca^{2+}-induced intermyocyte uncoupling seem to be a crucial common pathway in

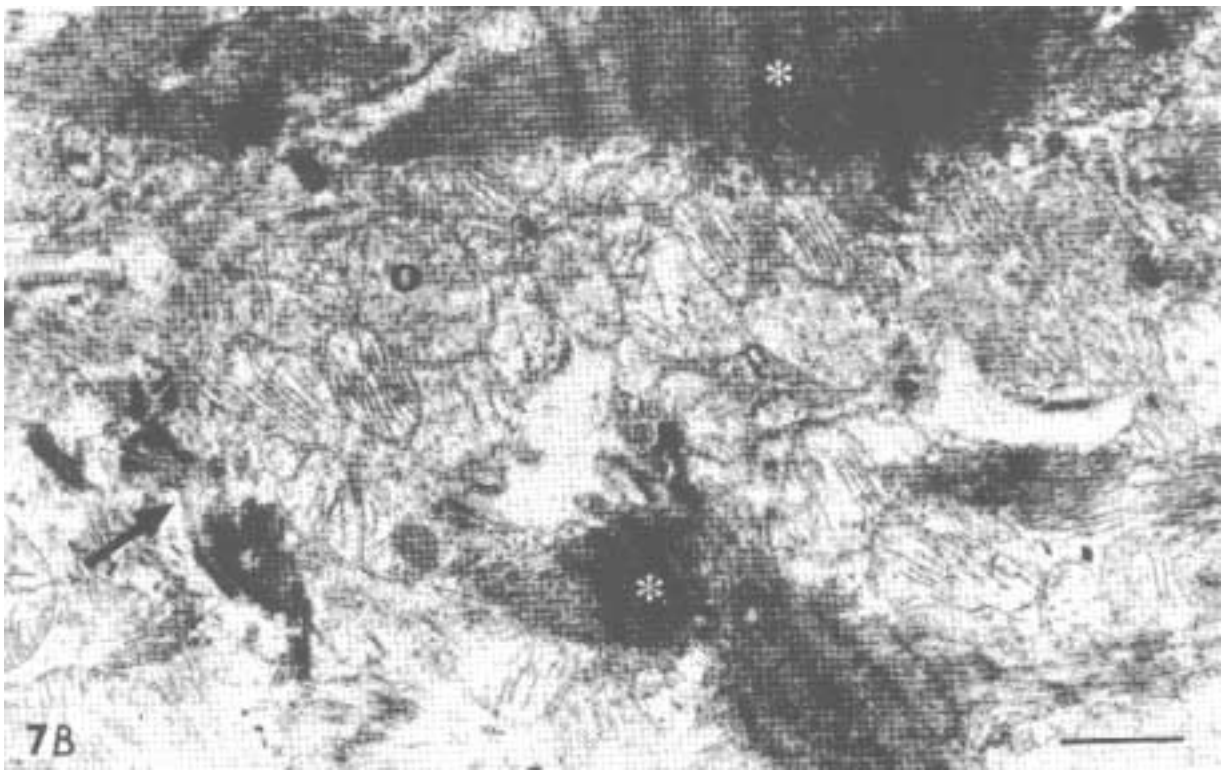

Fig. 7B. *Ultrastructure of the cardiomyocyte from fibrillating myocardium indicates irreversible injury characterized by loss of integrity of intermyocyte junctions (arrow), ruptured mitochondria (o), hypercontraction of sarcomeres (asterisks). Bar represents 1 μm.*

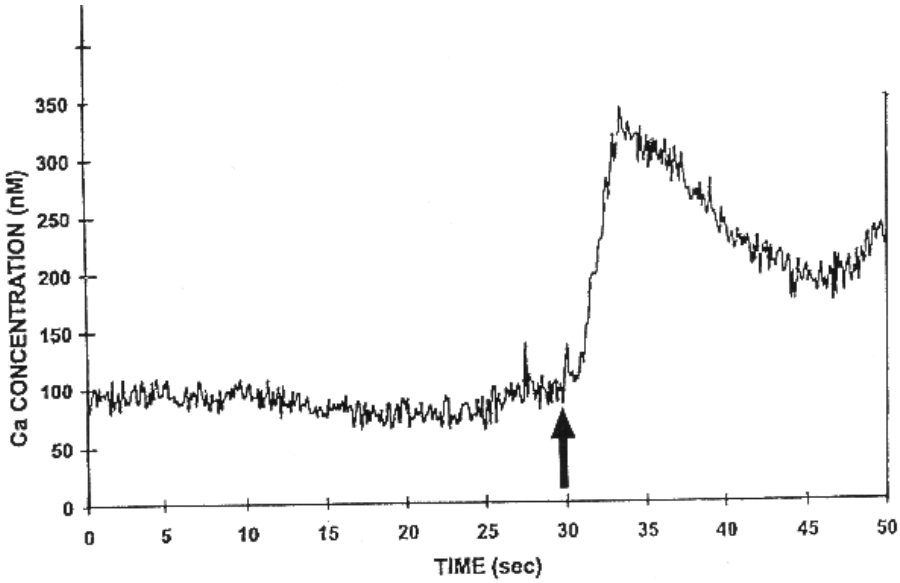

Fig. 8. *Intracellular Ca^{2+} concentration in cultured ventricular myocytes increased from 100 nmol/l to 350-200 nmol/l after exposure to a K^+-free solution (indicated by arrow).*

fibrillation occurrence. Conversely, the prevention or attenuation of Ca^{2+} overload with consequent protection of gap junction channels as well as direct upregulation of cell-to-cell coupling and synchronization by endogenous (Darrow *et al.* 1996) or by exogenous (Dhein *et al.* 1994) compounds may be a very promising target for antifibrillating/defibrillating drug therapy. This suggestion was also supported by findings that treatment with both an inhibitor of Na^+/Ca^{2+} exchanger and a Ca^{2+} channel blocker decreased the incidence of fibrillation.

Moreover, compounds which were shown to attenuate the Ca^{2+} overload (Manoach *et al.* 1997, Tribulová *et al.* 1999a, Tribulová and Manoach 1999) facilitated the conversion of fibrillation into a sinus rhythm.

In summary, our results have shown that perfusion of the isolated guinea pig heart with a K^+-deficient solution results in significant qualitative changes heterogeneously distributed within the myocardium. These are characterized by decreased enzyme activities, abolished immunodetection of connexin-43 and subcellular injury with impairment of intermyocyte coupling as well as arrhythmias and fibrillation. These changes are interrelated and they reflect electrolyte disturbances including a Ca^{2+} overload. We suggest that extensive Ca^{2+}-related alterations may account for both the initiation of arrhythmias and their degeneration into sustained ventricular fibrillation. The dispersion of intermyocyte uncoupling may be fundamental in this process. Prevention of the Ca^{2+} overload can protect the heart against ventricular fibrillation.

Acknowledgements

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