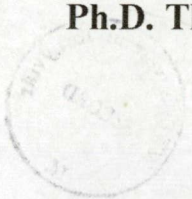
Cellular analysis of proarrhythmic and antiarrhythmic drug effects

Ph.D. Thesis



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2000

Contents

List of publications related to the subject of the Thesis

Full papers

I. Beáta Baláti, András Varró, Julius Gy. Papp: Comparison of the electrophysiological effect of cibenzoline, almokalant and amiodarone in isolated canine right ventricular trabecular muscle. (in Hungarian) *Cardiologia Hungarica* 3:19-23, 1996.

II. Beáta Baláti, Norbert Iost, Judit Simon, András Varró, Julius Gy. Papp: Analysis of the electrophysiological effects of ambasilide, a new antiarrhythmic agent, in canine isolated ventricular muscle and Purkinje fibers. *General Pharmacology* (In press).

III. Beáta Baláti, András Varró, Julius Gy. Papp: Comparison of the cellular electrophysiological characteristics of canine left ventricular epicardium, M cells, endocardium and Purkinje fibers. *Acta Physiologica Scandinavica* 164:181-190, 1998.

IV. Beáta Baláti, András Varró, Julius Gy. Papp: Electrophysiological characterization of the M cells: their physiological, pharmacological and clinical significance. (in Hungarian) *Cardiologia Hungarica* 4:155-164, 1998.

V. Beáta Baláti, András Varró, Julius Gy. Papp: Pharmacological modification of the dispersion of repolarization in the heart: importance of the M cells. *Cardiovascular Drugs and Therapy* 13:491-505, 1999.

VI. András Varró, Beáta Baláti, Norbert Iost, János Takács, László Virág, David A. Lathrop, Csaba Lengyel, László Tálosi, Julius Gy. Papp: The role of I_{Ks} in dog ventricular muscle and Purkinje fiber repolarization. *Journal of Physiology* 523:67-81, 2000.

Quotable abstracts

VII. Beáta Baláti, Sahab Noori, András Varró, Julius Gy. Papp: The cellular electrophysiological effects of desethylamiodarone in dog cardiac ventricular muscle and Purkinje fibers. *Journal of Molecular and Cellular Cardiology* 28:A235, 1996.

VIII. András Varró, Miklós Németh, János Takács, Ottó Hála, Beáta Baláti, Julius Gy. Papp: Comparison of the cellular electrophysiological effects of amiodarone and dronedarone in canine ventricular muscle and Purkinje fibers. *Fundamental & Clinical Pharmacology* 13(Suppl 1):S35.4, 1999.

Summary

The most important findings presented in this Ph.D. thesis are the following:

1.) Almokalant, cibenzoline and chronic amiodarone treatment significantly lengthened the effective refracter period (ERP) at 1 Hz stimulation frequency. Chronic amiodarone administration depressed impulse conduction only at stimulation frequencies higher than 1 Hz which effect was intensified by faster stimulation. In contrast, cibenzoline depressed impulse conduction also at normal and slow heart rate, which effect may have harmful consequences by increaseing proarrhythmic risk. Cibenzoline markedly reduced the ERP shortening effect of cromakalim, indicating inhibition of the ATP-sensitive potassium channels that play an important role during cardiac ischaemia.

2.) Desethylamiodarone possesses significant acute electrophysiological effects, which are different in cardiac Purkinje and ventricular muscle fibers and similar to those observed earlier with the parent compound amiodarone. Despite its lack of iodinated substituents, dronedarone, a novel derivative of amiodarone, exerts similar acute electrophysiological effects to those of amiodarone, and this finding could account for its promising role in the treatment of ventricular arrhythmias.

3.) Ambasilide, a new investigational antiarrhythmic agent, in addition to its Class III antiarrhythmic effect which is characterized by the lack of reverse use-dependence, possesses also IB type antiarrhythmic properties. These actions of ambasilide are similar to those of amiodarone and suggest that because of its advantageous, „multifaced" electrophysiological profile, this agent may be a promising drug candidate for antiarrhythmic therapy.

4.) The existence of M cells in the canine left ventricle described earlier was confirmed. Important differences in some action potential parameters of M cells were revealed, namely the distribution of the maximal rate of depolarization and action potential amplitude values reflecting probably the inhomogeneity of the fast sodium current in these cells. It was demonstrated that M cells differ from Purkinje fibers in some aspects which were not obvious from previous investigations; 1. the early portion of the action potential duration (APD) restitution curve in M cells is more similar to that of endocardial and epicardial cells than to Purkinje fibers, 2. the potential range of the plateau phase in the M cell action potential is also more similar to that of endocardial and epicardial cells than to Purkinje fibers, 3. the pharmacological response of M cells to tetrodotoxin and pinacidil resembles more that of the endocardial and epicardial cells than the effect of these agents on Purkinje fibers.

5.) Comparing the effects of two recently developped selective blockers of the slow component (I_{Ks}) of I_K to those produced by two recognized selective blockers of the rapid component (I_{Kr}) on normal dog ventricular muscle and Purkinje fiber action potential repolarization, it was found that in contrast to specific I_{Kr} bolckers, specific inhibitors of I_{Ks} do not lengthen APD in either type of cardiac tissues. However, when APD is abnormally lengthened by other means, the effect of selective I_{Ks} blockers on repolarization increases substantially resulting in an excessive prolongation of the ventricular muscle APD.

1. Introduction

Cardiac arrhythmias represent a major problem in clinical practice: for example, atrial fibrillation affects 1 million patients, and ventricular fibrillation, which is the most common cause of sudden cardiac death (in 70 to 80% of cases), kills 400,000 people every year in the USA alone (1). Accordingly, cardiac arrhythmias represent a major area of cardiovascular research. Drug therapy has traditionally been the mainstay of treatment for both ventricular and supraventricular arrhythmias. Nevertheless, serious side effects may considerably limit the use of several antiarrhythmic agents. Ventricular fibrillation was well recognized as being the primary cause of sudden cardiac death as early as in the early twentieth century (2), and in a classical series of experiments it was shown that ventricular fibrillation can be induced by critically timed electrical stimuli (3). This led to the "electrical accident" theory of sudden death, according to which premature ventricular contractions (PVCs) falling in a critical phase of the cardiac cycle induce ventricular fibrillation and thereby sudden death. In concordance with these findings, through the 1970's, antiarrhythmic drug development focused on the development of more effective oral agents for the suppression of PVCs, as quantified by 24-hour ambulatory Holter monitoring. This effort culminated in the development of two highly-effective class IC agents, encainide and flecainide, in the early 80's (4,5). The Cardiac Arrhythmia Suppression Trial (CAST) (6) was designed to confirm the ability of PVC-suppressing class IC drugs to prevent sudden death in post-myocardial infarction patients with frequent PVCs. CAST was terminated prematurely when it was shown that the treatment group had a significant excess in mortality, due largely to an increase in the incidence of sudden death (7). This result was unexpected and shocking, and motivated a fundamental reevaluation of the strategy of drug treatment in ventricular tachyarrhythmias. Initially, the negative outcome of CAST was thought to reflect the particular properties of the CAST population, however, a subsequent analysis of controlled studies of quinidine in atrial fibrillation also pointed to mortality promotion in the relatively less sick atrial fibrillation population (7). Before the outcome of these results, the vast majority of antiarrhythmic compounds abolished arrhythmia by a mechanism of slowing or blocking impulse conduction due to a depression of the fast inward sodium current. These studies, however, underscored the importance of the proarrhythmic effects of Class I antiarrhythmic drugs and as a consequence, interest has been significantly lost in them. The disaffection with Class I drugs

for the prevention of sudden death has led the pharmaceutical industry to attempt to develop other agents, such as Class III compounds, that selectively prolong myocardial refractoriness without slowing intracardiac conduction, and at present, class III antiarrhythmic agents are favoured increasingly to treat patients with serious tachycardias. The use of the dextro-isomer of sotalol, which is a pure Class III compound, was tested in postinfarction patients with left ventricular dysfunction in the SWORD (Survival With Oral d-Sotalol) trial (8). Despite the favourable antiarrhythmic effect, however, also this trial was stopped prematurely because patients on d-sotalol demonstrated a statistically significant increased mortality and sudden death risk. The adverse effect of the drug on mortality could be due to its bradycardia-dependent proarrhythmic ("torsadogenic") effect, and most other pure Class III compounds might have a similar harmful effect. The outcome of SWORD had a great impact on the development of new antiarrhythmic drugs. Numerous pure Class III compounds under investigation have been discontinued from clinical development (9). Emphasis has therefore been shifting to the compounds with a multifaceted ("hybrid") pharmacological profile (10,11) (with multiple molecular targets in the framework of the Sicilian Gambit (12). Many investigators now believe that the ideal antiarrhythmic drugs for the future should share the complex profile of amiodarone, which is regarded nowadays as the most effective drug available for cardiac rhythm disturbances, exhibiting a uniquely complex spectrum of electropharmacological actions, with properties of all four antiarrhythmic classes (13). Accordingly, development of such drugs is expected to offer favourable new options for antiarrhythmic drug therapy.

In accordance with the above, one of the goals of my PH.D. project was to investigate the cellular electrophysiological mechanisms of various antiarrhythmic drugs that act by lengthening the cardiac repolarization (notably cibenzoline, almokalant, amiodarone, desethylamiodarone, dronedarone, ambasilide, d-sotalol, E-4031, chromanol 293B, L-735,821) in isolated dog right ventricular papillary muscle and Purkinje fibers. Particular attention has been paid to their antiarrhythmic and proarrhythmic properties, rate dependence over a wide range of stimulation frequencies, the impact of their selectivity profile on their rate-dependent characteristics and finally their restitution kinetics.

Differential diagnosis of cardiac arrhythmias requires an understanding of basic mechanisms and establishment of mechanism-specific electrophysiological criteria. Both in turn depend on our knowledge of the basic electrophysiological characteristics of the cells and

tissues of the heart and the extent to which heterogeneity or specialisation exists. Our ability to design specific drug treatments also depends on our understanding and awareness of differences in the pharmacological responsiveness of diverse cell types within the heart. Until recently, the ventricular muscle, which constitutes the vast majority of the mammalian heart, was thought to be relatively homogeneous with respect to electrophysiological and pharmacological properties. This might have been related to the fact that most of the electrophysiological and pharmacological knowledge concerning the ventricular myocardium stemmed from studies using endocardial preparations and Purkinje fibers, which are specialized for impulse conduction. Data obtained from endocardium were often generalized and considered to be representative of the ventricular myocardium as a whole. Recent studies, however, have revealed important regional differences in the electrophysiology and pharmacology of the ventricular myocardium in mammalian hearts (14,15) and provided data indicative of the existence of at least four functionally distinct cell types in the ventricles, including epicardial, midmyocardial (M), endocardial and Purkinje cells. The midmyocardial or M cells, an electrophysiologically distinct population of cells in the deep subepicardial to midmyocardial layers of the canine ventricular free wall, were identified by Sicouri and Antzelevitch (16), and due to their longer repolarization characteristics they were found to have a significant role in creating enhanced dispersion of repolarization in the ventricular wall, thereby contributing importantly to arrhythmogenesis, in particular to intramural reentry and triggered activity. The characterization of the M cells in the ventricular wall of different mammalian species has prompted a reevaluation of some existing concepts relating to the electrophysiology, pharmacology and pathophysiology of the ventricles of the heart. Since the electrophysiological and pharmacological properties of M cells have not been fully elucidated, another major goal of my work was to provide further *in vitro* characterization of the existing differences among the four tissue types of the dog ventricle over a wide range of stimulation cycle lengths, and furthermore, to compare the pharmacological response of these cell types to different compounds, in the first instance to the sodium channel blocker tetrodotoxin, the calcium channel blocker nifedipine and the ATP-sensitive potassium channel activator pinacidil. Since the majority of studies thus far available on this topic suggest that M cells bear outstanding resemblance with Purkinje fibers and share only minor similarities with ventricular muscle fibers in the epicardium and endocardium, the focus of the investigations was set

especially on the electrophysiological and pharmacological differences between midmyocardial and Purkinje cells of the canine ventricle.

The delayed rectifier potassium current (I_K) is one of the most important transmembrane ionic currents controlling repolarization in mammalian ventricular muscle (17). In most species I_K consists of two components, I_{Kr} (rapid) and I_{Ks} (slow). These two components differ from each other with respect to their drug sensitivity, rectification and kinetic properties (18). The relative small density of I_{Ks} was proposed to be the main reason for the longer repolarization observed in M cells relative to epicardial and endocardial cells (19). Specific blockers of I_{Kr} have been widely shown to lengthen cardiac action potential duration (APD) and this is consistent with their strong antiarrhythmic potency. Despite their favourable antiarrhythmic effect, however, the already mentioned SWORD study (8) revealed an increased mortality in patients treated with the I_{Kr} blocking antiarrhythmic drug d-sotalol. The reason is complex, but the following features of these drugs must be involved in the failure. First, pure Class III drugs (i.e. those that block I_{Kr} selectively) increase the inhomogeneity of repolarization and consequently that of the refractoriness. Second, the reverse use-dependent effect of these drugs (i.e. that they cause greater prolongation of the APD at slow versus rapid rates of stimulations) is also disadvantageous because at slow heart rate it may cause early afterdepolarizations (EAD) and consequently Torsade de Pointes (TdP) type ventricular arrhythmias. This phenomenon has been demonstrated with various I_{Kr} blocking agents, like dofetilide, E-4031 etc. and was found to be especially pronounced in Purkinje fibers (20). The absence of specific blockers of I_{Ks} has made it until recently impossible to evaluate directly the physiological role of this transmembrane ionic current in cardiac repolarization. Direct examination of this current, however, should be of great significance since according to data stemmed from guinea pig myocytes, the specific block of I_{Ks} might increase APD and refractoriness in a frequency-independent manner, preventing thus the proarrhythmic reverse use-dependent effect of antiarrhythmic drugs that lengthen repolarization (21). On this basis, significant effort has been focused on the development of selective I_{Ks} blockers as potential antiarrhythmic agents with a more desirable profile of rate-dependent action and safety than currently available class III compounds. Chromanol 293B (22) and L-735,821 (23) have recently been reported to selectively block I_{Ks} in guinea pig cardiac myocytes, but their effects on cardiac action potential configuration have not been examined in detail. Possessing these I_{Ks} blocker compounds, the third important objective of the present study was to establish the role

of the two components of I_K in producing normal cardiac action potential repolarization, furthermore to compare the effect of the selective inhibition of these two components in dog ventricular muscle and Purkinje fiber preparations. Thus, we compared the effects of the mentioned I_{Ks} blockers to those produced by two recognized, selective I_{Kr} blockers (E-4031 and d-sotalol) in the two type of cardiac tissues.

2. Methods

2.1. Animals

Mongrel dogs of either sex (body weights 8-20 kg) were used for the study. The animals were untreated, except for the dogs used to study the chronic effect of amiodarone which were given 25 mg/kg/day amiodarone (Cordarone, Sanofi) per os for 4 weeks. The investigations were in conformity with the *Guide for the care and use of laboratory animals* published by the US National Institutes of Health (NIH publication No 85-23, revised 1985).

2.2. Preparations

Endocardial preparations (obtained from papillary muscles and ventricular trabecular muscles), as well as epicardial and midmyocardial tissues were isolated from the left ventricle of hearts removed from anaesthetized (sodium pentobarbital 30 mg/kg iv.) mongrel dogs of either sex. The preparations used for studying M cells were obtained by razor blade shavings made parallel to the surface of the ventricular free wall according to the method described by Sicouri & Antzelevitch (16). Briefly, the left ventricle was cut from the base to the apex with scissors,and small cubes of transmural slices (= 1.5 x 1.0 x 1-1.6 cm) were made at different locations of the anterobasal and anteroapical surfaces also with scissors applied to one edge of the incision. The slices of ventricular fee wall were then carefully cut by razor blade shavings, to obtain final preparations which were 10 to 15 mm long, 10 mm wide and 1 mm thick. M cells were recorded from slices located 2-5.2 mm from the epicardial surface. Because we found no major differences between the characteristics of papillary muscles and trabeculae, we grouped them together as endocardium in the presentation of the results. Free running false tendons of Purkinje fibers were excised from the left ventricle of the same hearts. The

preparations were placed in a tissue bath and allowed to equilibrate for at least 2 hours while superfused with oxygenated (95 % O_2 : 5 % CO_2) Tyrode's solution (flow = 4-5 ml/min) warmed to 37°C (pH 7.3 ± 0.5) and containing (in mM/l) NaCl 123, KCl 4.7, $NaHCO_3$ 20, $CaCl_2$ 1.8, $MgCl_2$ 0.8 and D-glucose 10. Preparations were oxygenated also in the tissue bath directly.

2.3. Action potential recordings

The experiments were carried out by applying standard intracellular microelectrode technique, except for the ones examing the effect of almokalant, cibenzoline and chronic amiodarone treatment, in which extracellular electrophysiological recordings were used.

Standard intracellular microelectrode technique: During the equilibration period the tissues were stimulated at a basic cycle length (BCL) of 1000 ms. Electrical pulses of 2 ms in duration and twice diastolic threshold in intensity (S_1) were delivered through Teflon-coated bipolar silver electrodes to the preparations. Transmembrane potentials were recorded from one or more sites with the use of glass capillary microelectrodes filled with 3 M KCl (tip diameter < 1 μM, resistance 10 to 25 MΩ). The microelectrodes were coupled through an Ag-AgCl junction to the input of a high-impedance, capacitance-neutralizing amplifier (Biologic VF 102). The first time derivative of the upstroke of the action potential (AP) was obtained using an electronic differentiator (Biologic DV-140), the output of which was linear between 100 and 1,000 V/s. Intracellular recordings were displayed on a storage oscilloscope (Tektronix 2232) and led to a computer system (HSE APES) designed for on-line determination of the following parameters: resting membrane potential (RP), conduction time (CT), action potential amplitude (APA), action potential duration at 50 % and 90 % repolarization (APD_{50}, APD_{90}) and the maximal rate of rise of the action potential upstroke (V_{max}). In the case of papillary muscles, recordings were always made from the apical region, known to be devoid of Purkinje fibers. Experiments were not started until the preparations were fully recovered and displayed stable electrophysiological characteristics. We applied the following types of stimulation in the course of the experiments: stimulation with a constant cycle length of 1000 ms; stimulation with different constant cycle lengths ranging from 300 to 10,000 ms (or to 5,000 ms in the case of Purkinje fibers in order to avoid spontaneous diastolic depolarization at cycle length of 10,000 ms). To determine the restitution of action potential characteristics, extra test action

potentials were elicited using single test pulses (S_2) in a preparation driven at a BCL of 1000 or 500 ms, depending on the experiment. The S_1 - S_2 coupling interval was increased progressively from the end of the refractory period. The effective refractory period was defined as the longest S_1 - S_2 interval at which S_2 failed to elicit a propagated response. The diastolic intervals preceding the test action potential were measured from the point corresponding to 90 % of repolarization of the preceding basic beat to the upstroke of the test AP and were increased progressively. To study the onset kinetics of V_{max}, preparations were continously stimulated at BCL of 1000 ms. The stimulation was interrupted for 1 minute and then a train of 40 beats stimuli was applied with BCL of 400 ms.

Extracellular electrophysiological recordings: We used only papillary muscle for this type of experiments. The impulse conduction time and the effective refractory period (ERP) were measured. To measure conduction time, bipolar extracellular platinum electrodes (diameter = 0.1 mm) were placed on the surface of the right ventricular wall along the direction of the trabecular muscle fibers, 10-12 mm away from the stimulating electrodes, and the propagated biphasic action potentials were recorded extracellularly. The propageted extracellular action potentials appeared at the recording electrode, and the time difference between the sign of the stimulus artifact and the extracellular potential was determined as conduction time. The extracellular action potentials were amplified with an amplifier (Eltron GMK) and were displayed on the screen of an oscilloscope (Medicor VM62A). The ERP was determined at three times the threshold strength using twin impulses with gradually increasing the coupling intervals. The stimulation frequency was varied between 3.5 and 0.5 Hz.

2.4. Drugs

Drugs were diluted in normal Tyrode's solution to obtain the following final concentrations: 10 μM/l cibenzolin (UPSA), 100 nM/l almokalant (ASTRA, Sweden), 25 mg/kg/day for 4 weeks (chronic treatment) + 5 μM/l amiodarone (Sanofi, France) into the tissue bath, 10 μM/l desethylamiodarone (Sanofi, France), 10 μM/l dronedarone (SR-33589) (Sanofi, France), 10 μM/l ambasilide (Knoll AG, Germany), 30 μM/l d-sotalol (Bristol-Myers Squibb, UK), 10 μM/l chromanol 293B (Hoechst AG, Germany) 100 nM/l L-735,821 (MSD, USA), 1 μM/l E-4031 (GYKI, Budapest), 10 μM/l pinacidil (GYKI, Budapest), 2 μM/l

tetrodotoxin (Sigma, St. Louis, USA), 2 μM/l nifedipine (Sigma, St. Louis, USA). All measurements were begun 15-30 min after the APD and V_{max} reached stable values.

2.5. Statistics

All data are expressed as mean ± SEM. Statistical analysis was performed using Student's t test for paired or unpaired data, as indicated and the nonparametric form of analysis of variance coupled with the Mann-Whitney and Bonferroni procedures. The results were considered to be significant at $P < 0.05$ level.

3. Results

3.1. Electrophysiological effects of cibenzoline, almokalant and chronic amiodarone treatment on canine right ventricular trabecular muscle

Since the publication of the CAST study (6), significant interest has been focused on antiarrhythmic drugs which delay repolarization in cardiac muscle. Antiarrhythmic drugs that inhibit I_K (Class III drugs) act to suppress arrhythmias primarily by lengthening the refracter period of atrial and ventricular myocardium and as a consequence they are effective in the termination as well as prevention of both supraventricular and ventricular reentrant tachycardia. In accordance with the above, we studied and compared the cellular electrophysiological effects of three antiarrhythmic drugs (cibenzoline, almokalant and amiodarone) which delay repolarization, in isolated dog right ventricular trabecular muscle by applying extracellular electrophysiological technique (24). The effects of 100 nM/l almokalant, 10 μM/l cibenzoline and chronic amiodarone treatment (p.o. 25 mg/kg/day for 4 weeks + 5 μM/l amiodarone in the tissue bath) were measured on the ERP and impulse conduction time at a wide range of stimulation frequencies (0.5 - 3.5 Hz). The possible effects of the drugs on the ATP-sensitive potassium current ($I_{K\text{-}ATP}$) were tested by activating the $I_{K\text{-}ATP}$ channels with 2 μM/l cromakalim, the effect of which results in the opening of these channels and as a consequence in the shortening of the ERP. We found that all three drugs significantly lengthened ERP at 1 Hz stimulation frequency. The impulse conduction time was not changed by almokalant at any of the stimulation frequencies. In contrast, cibenzoline significantly

potentials were elicited using single test pulses (S_2) in a preparation driven at a BCL of 1000 or 500 ms, depending on the experiment. The S_1 - S_2 coupling interval was increased progressively from the end of the refractory period. The effective refractory period was defined as the longest S_1 - S_2 interval at which S_2 failed to elicit a propagated response. The diastolic intervals preceding the test action potential were measured from the point corresponding to 90 % of repolarization of the preceding basic beat to the upstroke of the test AP and were increased progressively. To study the onset kinetics of V_{max}, preparations were continously stimulated at BCL of 1000 ms. The stimulation was interrupted for 1 minute and then a train of 40 beats stimuli was applied with BCL of 400 ms.

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increased conduction time (29.8 ± 7.3%; n = 8; $p < 0.05$) at low stimulation frequency (05 Hz), which effect was enhanced by faster rate of stimulation (3.5 Hz = 49.4 ± 9.3%; n = 8; $p < 0.05$) (Fig. 1/A). Chronic amiodarone treatment depressed impulse conduction only at stimulation frequencies higher than 1 Hz which effect was intensified by faster stimulation (1 Hz = 7.5 ± 1.5%; n = 22; $p < 0.05$; 3.5 Hz = 32.9 ± 1.6%; n = 22; $p < 0.05$) (Fig. 1/B). These results suggest the use-dependent inhibition of the fast sodium channels, although there is an important difference between the mode of inhibition of the two drugs: in contrast to amiodarone, cibenzoline depressed impulse conduction also at normal and slow heart rate, which effect seems to be undesirable from therapeutic point of view as it can increase proarrhythmic risk.

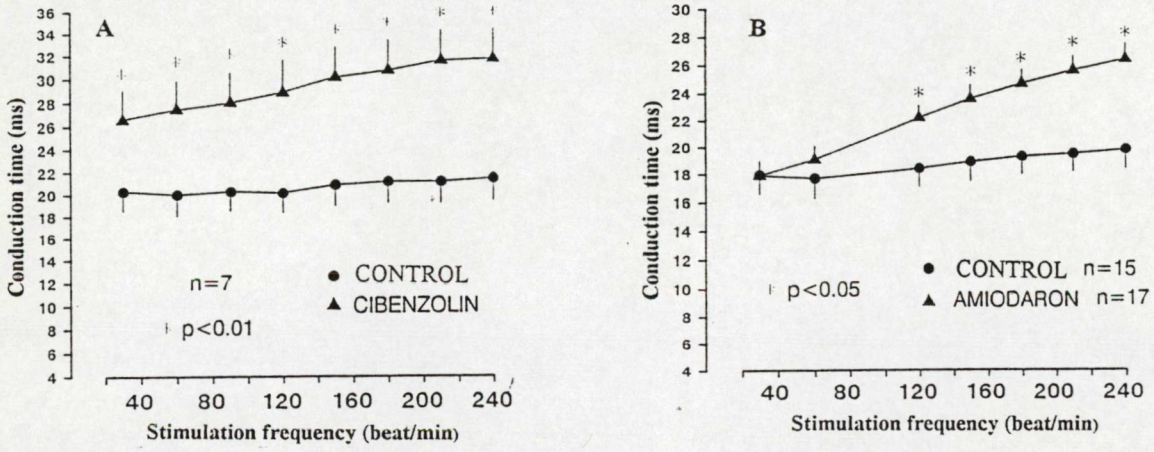


Figure 1. Frequency-dependent effect of cibenzoline (10 μM/l) (**Panel A**), and chronic amiodarone treatment (25 mg/kg/day, 4 weeks + 5 μM/l amiodarone in the tissue bath) (**Panel B**) on the impulse conduction time in dog ventricular muscle. Mean ± SEM values are shown. Asterisks denote $P < 0.05$ versus control.

Studying the possible effects of the drugs on the $I_{K\text{-}ATP}$, it was found that cibenzoline markedly reduced the ERP shortening effect of cromakalim (control = -19.7 ± 3.5%; cibenzoline = -5.4 ± 1.3%; n = 10; $p < 0.05$) suggesting the inhibition of the ATP-sensitive potassium channels that play an important role during cardiac ischaemia (Fig. 2). No such effect was observed with almokalant and amiodarone.

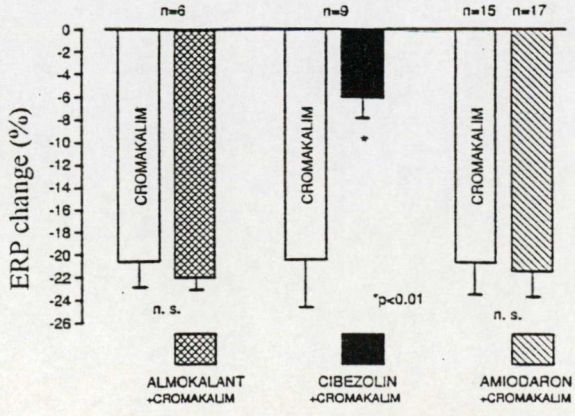


Figure 2. Effects of almokalant (100 nM/l), cibenzoline (10 μM/l) and amiodarone (25 mg/kg/day, 4 weeks + 5 μM/l amiodarone in the tissue bath) on the cromakalim-induced effective refractory period (ERP) shortening. The ERP change represents the percent difference between ERP values in the absence and presence of the drugs. Mean ± SEM values are shown. Asterisks denote $P < 0.05$ versus control.

3.2. Comparison of the cellular electrophysiological effects of desethylamiodarone, dronedarone and amiodarone in canine ventricular muscle and Purkinje fibers

Amiodarone is regarded nowadays as the most effective drug available for cardiac rhythm disturbances, exhibiting a uniquely complex spectrum of electropharmacological action, the properties of which belong to all four antiarrhythmic classes (13). It has been proven by large clinical trials that it has an extremely potent antiarrhythmic activity, and in addition to its ability to suppress effectively both ventricular and supraventricular arrhythmias, some studies showed that it was also able to reduce significantly mortality due to sudden cardiac death (13). It is well-known that the effects of acute and chronic administration of amiodarone are considerably different (13,25,26). On the basis of earlier experiments it can be expected that the cardiac electrophysiological effects of chronic amiodarone treatment are, at least partly, due to the formation and accumulation of an active metabolite, N-desethylamiodarone in the plasma and tissues (27). Since the electropharmacological characteristics of this important active metabolite have not been entirely elucidated, I have examined the acute *in vitro* effects of 10 μM/l desethylamiodarone over a wide range of stimulation cycle lengths in canine cardiac Purkinje and ventricular muscle fibers, and compared them with those of amiodarone (28). It is known that APD in the Purkinje fibers is normally longer than that in the ventricular muscles. I found that in Purkinje fibers at stimulation cycle length of 1000 ms (1 Hz), desethylamiodarone produced significant shortening in APD at both 50% and 90% repolarization (from APD_{50} = 318.0 ± 21.6 ms to APD_{50} = 231.7 ± 19.4 ms; $n = 5$; $p < 0.05$ and from APD_{90} = 413.2 ± 30.5 ms to APD_{90} = 362.0 ± 28.1 ms; $n = 5$; $p < 0.05$) and it also shortened APD_{90} at different constant cycle lengths and at abrupt changes in stimulation cycle length (restitution) as well. In ventricular muscle, however, desethylamiodarone did not prove to change APD neither at stimulation cycle length of 1000 ms, nor at different constant cycle lengths. The observed acute effect of the metabolite on APD ressembles that of amiodarone which due to its acute and especially chronic effect on repolarization is regarded to be at present the only antiarrhythmic drug, that act to reduce transmural dispersion of repolarization (TDR) at all stimulation

frequencies and therefore contributing to an overall electrical stability in the heart (25,26). Studying the effect of desethylamiodarone on the maximal rate of rise of depolarization, we observed a marked use-dependent depression of V_{max} in Purkinje fibers (at BCL of 400 ms = -28.4 ± 6.6%; n = 5; $p < 0.05$) with a recovery time constant of τ = 341.85 ± 81.6 ms (n = 5), that appears to be similar to that of amiodarone. Examing the same parameter in ventricular muscle, desethylamiodarone exerted moderate use-dependent depression of V_{max} (at BCL of 400 ms = -14.3 ± 9.4%; n = 5). The use-dependent effect on V_{max} (i.e. the largest depressant effect occurred at the shortest stimulation cycle length applied) suggests the depression of inactivated fast sodium channels, and this is consistent with a Class IB antiarrhythmic effect, i.e. with a relatively fast recovery time constant from sodium channel block (29). These results demonstrate that desethylamiodarone possesses significant acute electrophysiological effects, which proved to be different in cardiac Purkinje and ventricular muscle fibers and similar to those observed earlier with the parent compound amiodarone.

Despite its efficacy, however, amiodarone causes various extracardiac side effects which limit its usefulness. Thus great effort has been made in order to develop new compounds with similar electrophysiological profile, but lacking side effects. Dronedarone (SR-33589), a novel derivative structually related to amiodarone, but without iodine substituents, is such a compound; it has been found to be effective in various *in vivo* experimental arrhythmia models (30). Our aim was to characterize the acute *in vitro* cellular electrophysiological effects of dronedarone and to compare them with those of amiodarone (31). The experiments were carried out in canine papillary muscles and Purkinje fibers. Dronedarone (10 μM/l), like amiodarone (10 μM/l), did not significantly change or only slightly lengthened APD at BCL of 1000 ms in papillary muscle, but shortened the same parameter significantly in Purkinje fibers (dronedarone: 308.6 ± 12.4 to 283.8 ± 12.4 ms; n = 6; $p < 0.01$ and amiodarone: 307.0 ± 16.5 to 279.7 ± 11.9 ms; n = 7; $p < 0.01$). Both dronedarone and amiodarone exerted use-dependent IB type V_{max} block and suppressed EADs induced by dofetilide + BaCl2 in Purkinje fibers. It was concluded that despite its lack of iodinated substituents, dronedarone exerts similar acut electrophysiological effects to those of amiodarone, and this finding could account for its promising role in the treatment of ventricular arrhythmias.

3.3. Electrophysiological effects of ambasilide, a new antiarrhythmic agent, in canine isolated ventricular muscle and Purkinje fibers

Ambasilide (LU 47710) is a novel Class III agent, the chemical structure of which differs from that of sotalol and other methanesulfonyl benzamides recently synthesized and being investigated for Class III electrophysiological properties. On the basis of data from the literature, multiple pharmacological actions of ambasilide can be presumed (32,33,34). The aim of the study was to determine the *in vitro* rate-dependent cellular electrophysiological effects of ambasilide (10 and 20 μM/l), in canine isolated ventricular muscle and Purkinje fibers applying standard microelectrode technique.

The effects of 10 and 20 μM/l ambasilide on the action potential parameters of canine ventricular muscle and Purkinje fibers at a BCL of 1000 ms are summarized in Table 1.

Table 1. Effects of ambasilide on canine action potential parameters at cycle length of 1000 ms

Ventricular muscle	10 μM/l ambasilide (n=8)		20 μM/l ambasilide (n=8)	
	Control	Drug	Control	Drug
RP	-88±0.6	-90±1.1	-85±0.9	-85±0.6
APA	111±1.6	112±1.6	106±1.1	105±1.1
APD_{50}	219±10.4	254±13.3*	204±5.6	228±5.9*
APD_{90}	261±8.1	323±12.9†	242±5.7	289±6.6†
V_{max}	219±29.2	208±31.4	230±9.5	219±9.2

Purkinje fiber	10 μM/l ambasilide (n=8)		20 μM/l ambasilide (n=8)	
	Control	Drug	Control	Drug
RP	-89±1.0	-89±1.9	-87±0.6	-85±0.8
APA	112±3.5	112±2.0	117±1.2	113±2.1
APD_{50}	193±16.5	208±20.4	236±10.6	145±4.6†
APD_{90}	275±16.6	369±19.5†	321±9.9	361±7.2*
V_{max}	416±14.0	419±22.3	445±14.6	413±15.8

* $p < 0.05$; † $p < 0.001$

Ambasilide (10 μM/l) lengthened APD_{50} and APD_{90} significantly in papillary muscles without causing considerable change in the action potential parameters. In Purkinje fibers the same concentration increased only APD_{90} significantly. The effects of 20 μM/l ambasilide were similar to those of 10 μM/l in ventricular muscle. In the case of Purkinje fibers, however, there were important differences between the effects of the two concentrations on APD_{50} and APD_{90}. A lengthening of APD_{90} was observed in the presence of both concentrations of the drug, but while 10 μM/l ambasilide produced a marked prolongation (25.5% ± 1.9; $p < 0.01$; $n = 6$), 20 μM/l exerted a smaller effect (11.3% ± 3.0; $p < 0.05$; $n = 6$). As concerns the effects on APD_{50} in Purkinje fibers, 10 μM/l ambasilide caused prolongation, while 20 μM/l exerted a significant abbreviation. These effects of 10 and 20 μM/l ambasilide exerted on dog ventricular muscle and Purkinje fiber action potential configurations are shown in Figure 3/A and B.

The rate-dependent effect of ambasilide on APD_{90} was also studied. 10 μM/l Ambasilide was found to produce a similar degree of increase in APD_{90} at CLs 400 (16.1 ± 3.2%; $p < 0.05$; n = 8) and 3000 ms (21.9 ± 2.9%; $p < 0.05$; n = 8) in papillary muscles (Fig. 4/C).

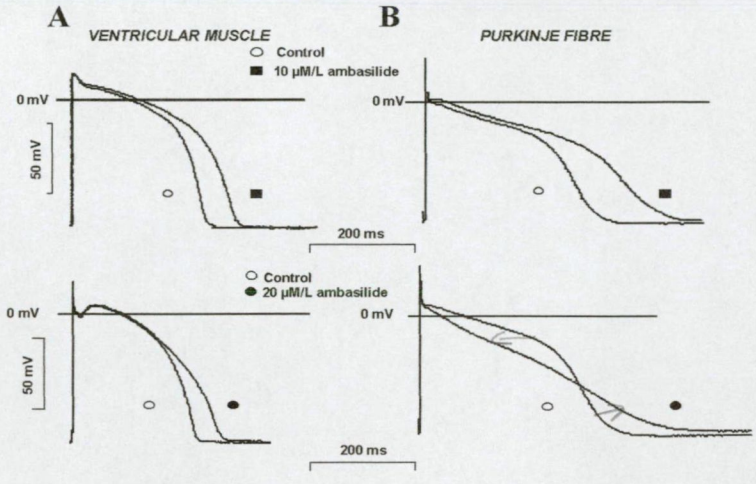


Figure 3. Action potential recordings from canine ventricular papillary muscles **(Panel A)** and Purkinje fiber strands **(Panel B)** before (control) and after superfusion for 40 minutes with 10 (top) and 20 μM/l ambasilide (bottom).

In contrast, the same concentration of ambasilide exerted a more pronounced increase in APD_{90} in Purkinje fiber preparations at long CLs; the difference tended to disappear at higher stimulation frequencies (CL = 400 ms) (Fig. 4/A). Examining the same effect of ambasilide in the concentration of 20 μM/l in ventricular muscle, we observed an even more homogenous prolongation of APD_{90} at all frequencies studied than with 10 μM/l (at CL = 400 ms: 16.9 ± 1.4%; $p < 0.05$; n = 9 and at CL = 3000 ms: 18.2 ± 1.5%; $p < 0.05$; n = 9) (Fig. 4/D). In Purkinje fibers, however, the higher concentration of the drug caused less change in APD_{90} than that observed with 10 μM/l. (Fig. 4/B).

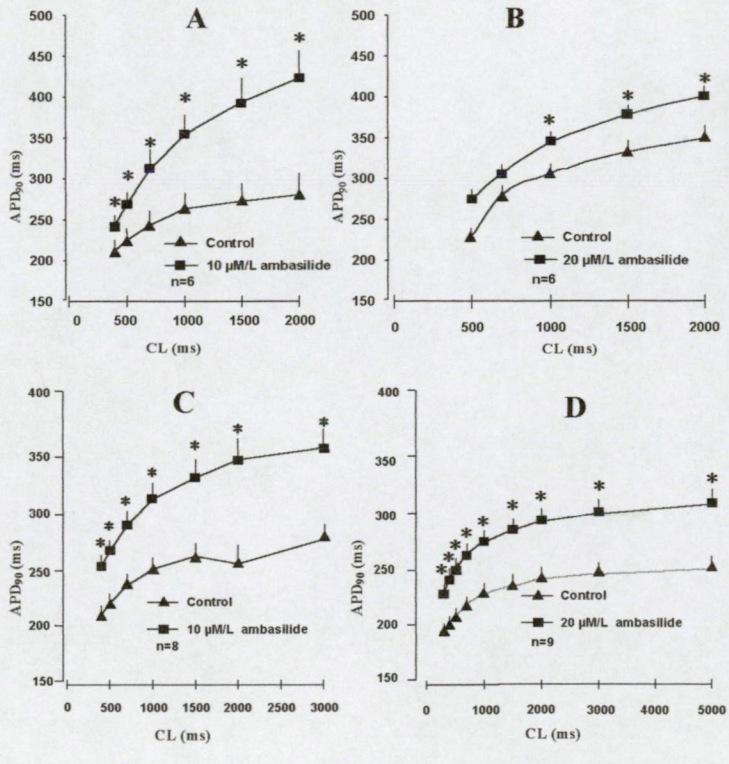


Figure 4. Rate-dependent effect of 10 and 20 μM/l ambasilide on APD_{90} in dog Purkinje fibers **(Panel A and B)** and in right ventricular papillary muscles **(Panel C and D)**. Mean ± SEM values are shown. Asterisk denotes $P < 0.05$ versus control.

We also studied the effect of ambasilide on the rate-dependent depression of V_{max}. In papillary muscle preparations the V_{max} depression caused by the drug (20

μM/l) was strongly cycle length dependent, i.e. significant effect was observed only within the CL range of 300-1000 ms (Fig. 5/A). The drug affected also the conduction time (CT) in papillary muscle. Similarly to the inhibitory effect on V_{max}, also this effect proved to be significant only within the CL range of 300-700 ms and the difference tended to disappear at CLs longer than 1000 ms (Fig. 5/B).

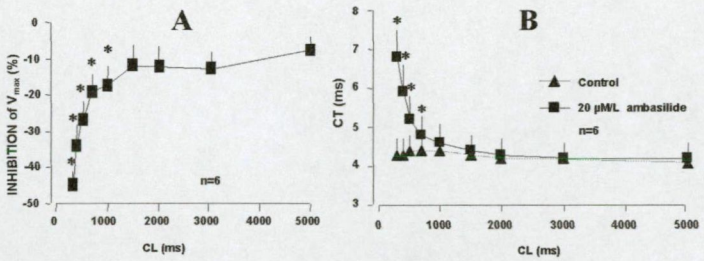


Figure 5. Rate-dependent inhibition of V_{max} caused by 20 μM/l ambasilide in canine ventricular muscle. The inhibition of V_{max} represents the percent difference between V_{max} values in the absence and presence of the drug. **(Panel A)** Rate-dependent effect of 20 μM/l ambasilide on conduction time (CT) in canine right ventricular papillary muscles. **(Panel B)** Mean ± SEM values are shown. Asterisk denotes $P < 0.05$ versus control.

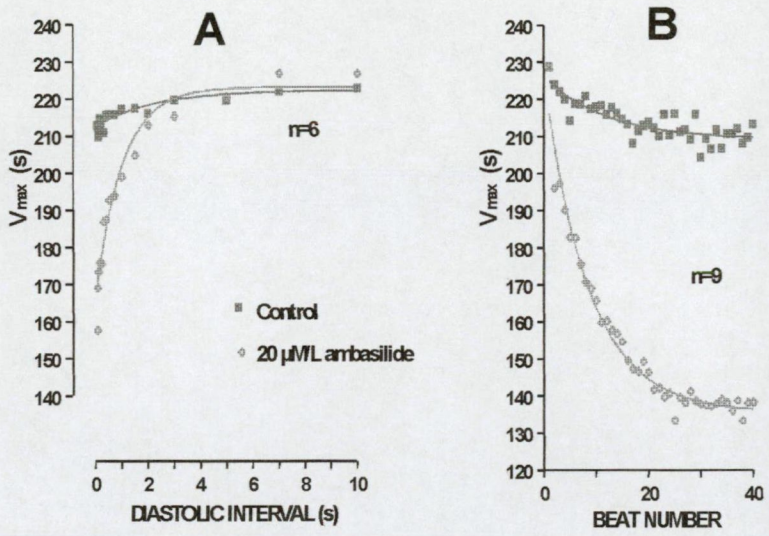


Figure 6. Effect of 20 μM/l ambasilide on the recovery of V_{max} **(Panel A)** and on the onset kinetics of V_{max} **(Panel B)** in canine right ventricular papillary muscles. Basic cycle length was 500 ms. In Panel A the ordinate indicates V_{max} values of the extrasystoles, elicited at progressively increasing diastolic intervals. The abscissa shows the diastolic intervals in seconds. Values are shown as mean. For the sake of clarity SEM is not indicated. (Range: 11.4 to 19.4 V/s.) In Panel B following a 1 min. stimulation free period, the V_{max} values of a train of 40 beats at a BCL of 400 ms are presented. The ordinate indicates the V_{max} values, the abscissa shows the number of the beats in the train. The range of SEM of V_{max} is 3.2 to 12.3 V/s.

As the determinants of V_{max} are known to differ between steady-state and non-steady-state stimulation conditions, we also examined the characteristics of restitution of this parameter in ventricular muscle preparations. Figure 6/A shows the recovery of V_{max} in 6 papillary muscle cells in the abscence and presence of 20 μM/l ambasilide. The restitution curves show that the drug slowed the recovery of V_{max} with a recovery time constant of $\tau = 1082.5 \pm 205.1$ ms ($n = 6$), which appears to be close to that reported with Class I/B agents. The onset kinetics of V_{max}

block induced by 20 μM/l ambasilide was also studied in dog right ventricular papillary muscle. (Fig. 6/B). Preparations were continously stimulated at BCL of 1000 ms. The stimulation was interrupted for 1 minute and then a train of 40 beats stimuli was applied with BCL of 400 ms (Fig. 6/B). In control conditions there was only a minor change (17.3 V/s) between the first and last V_{max} values in the train. In the presence of 20 μM/l ambasilide, however, a large (80.9 V/s) use-dependent V_{max} block developed with a rate constant of 8.9 ± 2.1 (n = 9). The onset kinetics of V_{max} block induced by ambasilide in this study was found to be intermediate between Class I/B and 1/C antiarrhythmic drugs and was similar to that reported of disopyramide and quinidine (35).

3.4. Comparison of the cellular electrophysiological characteristics and responsiveness to tetrodotoxin, nifedipine and pinacidil of canine left ventricular epicardium, M cells, endocardium and Purkinje fibers

One of the important goals of this study was to provide further *in vitro* characterization of the existing differences among the four tissue types of the dog ventricle over a wide range of stimulation cycle lengths, examing the electrophysiological and pharmacological heterogeneity caused by them, and the relevance of this heterogeneity in physiological and pathophysiological function. Since the majority of studies thus far available on this topic suggest that M cells bear outstanding resemblance with Purkinje fibers and share only minor similarities with epicardial and endocardial cells, I have focused my investigations especially on the electrophysiological and pharmacological differences between M and Purkinje cells of the canine ventricle (36). The experiments were carried out by applying standard intracellular microelectrode technique in isolated dog left ventricular preparations.

Action potentials recorded from preparations isolated from the epicardial, midmyocardial, endocardial regions and from Purkinje fibers of the canine left ventricle are illustrated in Figure 7. The four traces were recorded from the respective regions of the ventricle during stimulation of the preparations at a BCL of 300, 1000 and 5000 ms. Action potentials recorded from epicardial and M cells as well as from Purkinje fibers display a distinct early repolarization phase (phase 1) that is less obvious in endocardial cells. M cells differ from epicardial and endocardial cells but resemble Purkinje fibers with respect to phase 3 repolarization, showing a greater prolongation of the action potential with slowing of the stimulation rate. It is worth

mentioning that the plateau phase of the action potentials in Purkinje fibers developed at less positive potential (-5.4 ± 1.6 mV), than that in M cells (9.8 ± 1.3 mV), endocardial (13.3 ± 1.8 mV) or epicardial fibers (17.1 ± 1.1 mV).

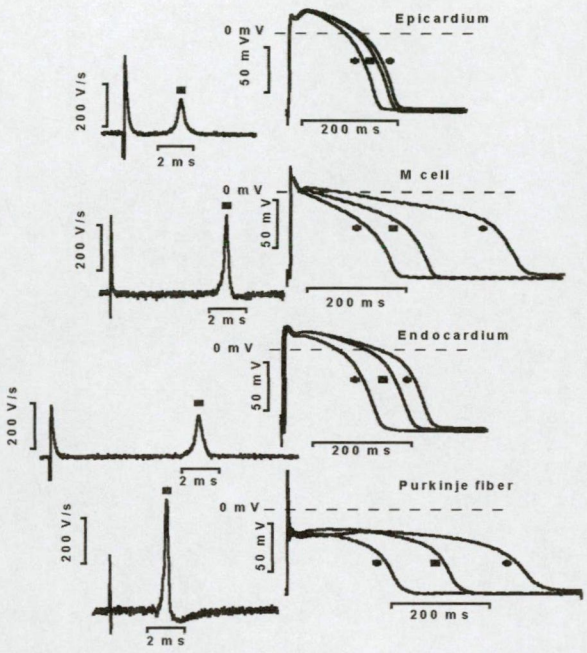


Figure 7. Transmembrane action potentials (right panel) recorded under steady-state conditions at BCLs of 300 (star), 1000 (square) and 5000 ms (diamond) from epicardium, M cells, endocardium and Purkinje fibers isolated from the canine left ventricle. The maximal rate of rise of the action potential upstroke (V_{max}) (left panel) recorded from the respective regions of the left ventricle at a BCL of 1000 ms (square).

Table 2 summarizes the action potential parameters of the four tissue types at the BCL of 1000 ms that approximates to normal human heart rate (60 beats/min). It was found that M cells and Purkinje fibers displayed a resting membrane potential more negative than that in epicardial and endocardial cells. Action potantial amplitude in Purkinje fibers was considerably higher than that observed in the different types of ventricular muscle fibers. The APD recorded in M cells at the BCL of 1000 ms was longer than that recorded from epicardial or endocardial cells although only the difference between M cells and epicardial cells proved to be significant, and was considerably shorter than the APD measured in Purkinje fibers. M cells displayed a V_{max} significantly greater than that of either epicardial or endocardial cells, but the V_{max} in M cells was less in magnitude than that in Purkinje fibers. Interestingly, the distribution of V_{max} values recorded in M cells demonstrated a marked variance in magnitude. Since endocardial and epicardial cells in normal conditions have a V_{max} value smaller than 300 V/s, we arbitrarily divided the V_{max} values of 37 individuel M cells into two subgroups, under and above 300 V/s. In 14 of 37 preparations it was found that the V_{max} value was greater than 300 V/s with a mean of 397.7 ± 18.5 V/s, while in 23 preparations V_{max} was measured less than 300 V/s with a mean of 214.8 ± 10.2. The distribution of V_{max} values in magnitude exhibited by M cells raised the question as to whether the other action potential parameters of the midmyocardial region can also be divided into two subgroups. It can be expected that parameters like V_{max}, which correlates with the

intensity of the sodium current during the action potential upstroke, may be considerably greater in M cells possessing a V_{max} higher than 300 V/s, but smaller in cells with a less pronounced maximal rate of rise of the action potential upstroke. In accordance with this expectation, we found that the action potential amplitude beared similarity with the variance of V_{max}. These features of action potential parameters in M cells are demonstrated in Table 3.

Table 2.

Action potential parameters of endocardial, M, epicardial cells and Purkinje fibers recorded from canine left ventricular preparations at basic cycle length of 1,000 ms

	ENDOCARDIUM (n = 28)	M CELL (n = 37)	EPICARDIUM (n = 29)	PURKINJE FIBER (n = 20)
RP (mV)	-84.3 ± 0.9	-86.5 ± 0.8	-84.2 ± 0.7	-89.6 ± 0.9 *†‡
APA (mV)	108.7 ± 1.5†	108.4 ± 1.5†	101.3 ± 1.2	124.6 ± 1.7 *†‡
APD_{90} (ms)	238.6 ± 5.0	258.7 ± 4.5†	222.1 ± 5.3	324.8 ± 15.4*†‡
V_{max} ($V\ s^{-1}$)	176.9 ± 7.0*	284.0 ± 17.5†	154.5 ± 6.6	505.0 ± 32.7*†‡

Values are mean ± SEM; Significance was determined by the nonparametric form of analysis of variance coupled with the Mann-Whitney and Bonferroni procedures. * $p < 0.01$ vs M cell; † $p < 0.01$ vs epicardium; ‡ $p < 0,01$ vs endocardium

	$V_{max} > 300$ V/s (n=14)	$V_{max} < 300$ V/s (n=23)
BCL of 1000 ms		
V_{max} (V/s)	397.7 ± 18.5*	214.8 ± 10.2
RP (mV)	-89.1 ± 1.2†	-85.0 ± 1.0
APA (mV)	114.2 ± 1.8*	104.8 ± 1.7
APD_{90} (mV)	260.8± 8.2	257.4 ± 5.2
BCL of 10,000 ms vs 1000 ms		
APD_{90} prolongation (%)	36.0 ± 6.4	47.7 ± 5.5

Table 3. Distribution of APD parameters in M cells from canine left ventricle preparations at cycle length of 1000 and 10,000 ms (* $p < 0.001$; † $p < 0.05$)

The main characteristic of M cells which differentiates them from the other ventricular cell types and makes them more similar to Purkinje fibers, is the more accentuated rate dependence of their action potential duration. To study this property, we stimulated the preparations at BCLs ranging from 300 to 10,000 ms (or to 5000 ms in the case of Purkinje fibers in order to avoid spontaneous diastolic depolarization at cycle length of 10,000 ms). At the BCL of 300

ms all the four cell types displayed relatively short action potentials of almost similar duration. With progressive slowing of the stimulation rate, APD of M cells was prolonged to a much greater extent than the APD of epicardial and endocardial cells. In the left ventricle, an increase of the BCL from 1000 to 10,000 ms caused a 41% increase in the APD_{90} of M cells, but an increase of only 14% in epicardial and 13% in endocardial fibers. In Purkinje fibers, a deceleration of rate from 1000 to 5000 ms produced a dramatic prolongation of APD similar to that of M cells (35%). Thus, the APD-rate relations are remarkably more prominent in M cells than those in epicardium or endocardium, and similar to those in Purkinje fibers, which are known to prolong more excessively relative to the other cell types when stimulation rate is slowed.

The characteristics of restitution of APD was also examined and compared in the four cell types. The APD restitution curves illustrated in Figure 8. show remarkable distinction between the restitution of APD in M cells and Purkinje fibers representing an important electrophysiological difference between the two types of fiber, which has not been described yet. The restitution curves in endocardial and epicardial fibers were relatively similar. The two exponentials fit on the average curves showed τ_{fast} = 60.9 ms, τ_{slow} = 32.7 s and τ_{fast} = 55.8 ms, τ_{slow} = 29.2 s, respectively. The corresponding amplitude values of the fit were A_{fast} = 41.1 ms, A_{slow} = 30.1 ms and A_{fast} = 20.1 ms, A_{slow} = 35.3 ms, respectively. The APD restitution of M cells is somewhat different from that of endocardium and epicardium. In these fibers the fast component of restitution had a τ_{fast} of 17.4 ms (A_{fast} = 29.9 ms) but, as considerable difference from epicardial and endocardial fibers, the amplitude of the slow component was relatively big (A_{slow} = 104.1 ms) with similar kinetic parameter (τ_{slow} = 23.8 s). Purkinje fibers, exhibit completely different course of APD restitution with high amplitudes (A_{fast} = 150.6 ms, A_{slow} = 77.6 ms) and relatively slow fast time constant of restitution (τ_{fast} = 127.6 ms) and relatively fast (τ_{slow} = 3.7 s) slow time constant of restitution.

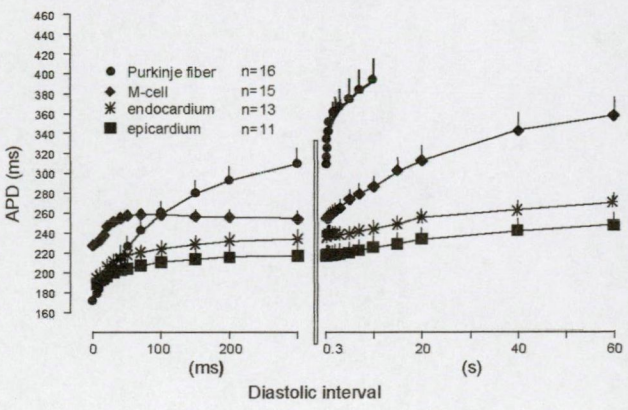


Figure 8. Restitution of APD_{90} in epicardium (square), endocardium (star), M cells (triangle) and Purkinje fibers (circle) isolated from the canine left ventricle. BCL was 1000 ms. The ordinate indicates APD_{90} values of the extrasystoles, elicited at progressively increasing diastolic intervals, in ms. The abscissa shows the diastolic intervals. In the early portion of restitution the time scale was expanded in order to illustrate better the initial phase of

restitution. Values are shown as mean ± SEM.

The change in the APD of extrasystoles elicited once after every 10th basic beat at early (20 ms) and at very late (60 s) diastolic intervals was also determined and compared in all four types of tissue. As Figure 9 shows, in case of extrasystoles elicited at early diastolic intervals (20 ms), the pattern of APD changes in endocardial, epicardial and M cells was strikingly different from that in Purkinje fibers; i.e., the APD of the premature beat shortened only moderately in endocardial, epicardial and M cells in comparison with the APD of the basic beat (15.6 ± 1.6 %, n = 24, 9.5 ± 1.9 %, n = 23, and 7.6 ± 2.0 %, n = 38, respectively), while the same parameter in Purkinje fibers showed a marked reduction (46.3 ± 1.6 %, n = 19).

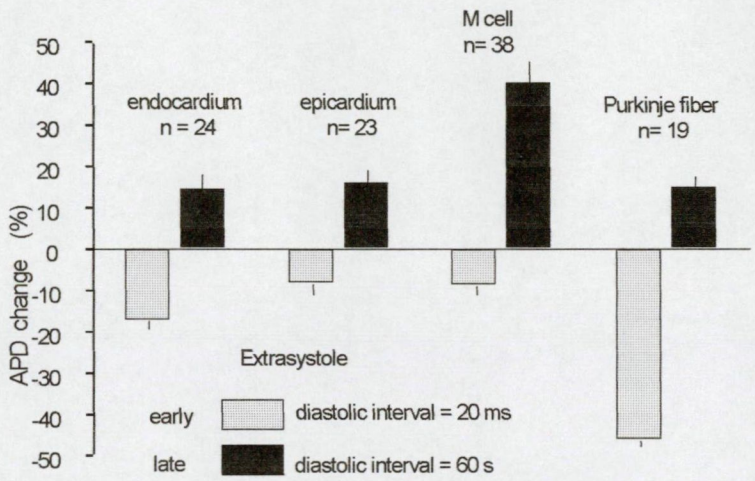


Figure 9. Cycle length dependent APD at early (20 ms) and late (60 s) diastolic intervals in endocardium, epicardium, M cells and Purkinje fibers of the canine left ventricle. Changes in the APD of extrasystoles elicited once after every 10th basic beat at early (grey columns) and late diastolic intervals (black columns) are expressed as a percentage of the APD of the basic beat.

This means that the early portion of the APD restitution curve in M cells is more similar to that of endocardial and epicardial cells than to Purkinje fibers. Regarding the change in the APD of extrasystoles elicited at late diastolic intervals (60 s), we found a great prolongation in M cells (40.0 ± 4.6 %, n = 38) and in Purkinje fibers (15.4 ± 1.7 %, n = 19), but only a slight increase in endocardial and epicardial cells (16.7 ± 2.6 %, n = 24 and 15.3 ± 3.1 %, n = 23, respectively). As far as the prolongation of APD of extrasystoles recorded in Purkinje fibers is concerned, we have to note that the longest diastolic interval in this type of tissue was 10 s to avoid automaticity. Considering the APD restitution curve in Purkinje fibers, it is likely that a quantitatively similar lengthening of APD to that of M cells could have been obtained, if the largest diastolic interval had been 60 s like in epicardial, endocardial and M cells.

Considering the electrophysiological heterogeneity among the four types of tissue, it can be expected that they respond differently to pharmacological interventions. Therefore we studied the pharmacological responses of the four tissue types, investigating the effects of tetrodotoxin

(TTX 2 μM/ l), nifedipine (2 μM/l) and pinacidil (10 μM/l) on the APD of the preparations at BCL of 1000 ms (Fig. 10.).

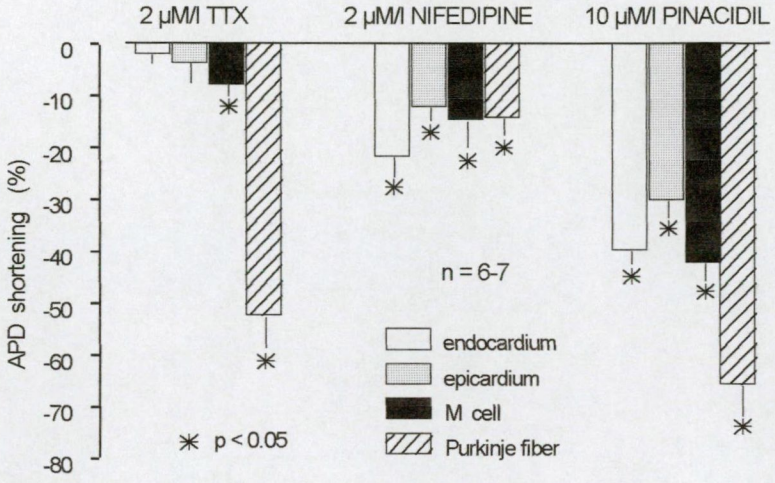


Figure 10. Block diagram illustrating the APD shortening effects of tetrodotoxin (TTX 2 μM/l), nifedipine (2 μM/l) and pinacidil (10 μM/l) on canine left ventricular endocardium (white), epicardium (grey), M cells (black) and Purkinje fibers (striped columns) at aBCL of 1000 ms. Changes in APD are expressed as percentage of control APD in the presence of the drugs. Vertical bars indicate SEM. * denotes $p < 0.05$ vs control.

It was found that after 10 minutes of exposure to 2 μM/l tetrodotoxin, APD was shortened in both M cells and Purkinje fibers significantly, whereas no significant changes in APD were observed in epicardium or endocardium after application of this agent. Despite the qualitatively similar effect of TTX on M cells and Purkinje fibers, it has to be emphasised that the degree of APD abbreviation was more accentuated in Purkinje fibers than in M cells (52.8 ± 5.5 %; n = 6; and 8.2 ± 4.2 %; n = 6; respectively), suggesting a weaker contribution of the sodium current to repolarization in M cells than in Purkinje fibers, findings making M cells to be more akin to epicardial and endocardial cells. The calcium channel blocker nifedipine produced a significant reduction in APD of all the four cell types, and the shortening effect caused by this drug did not show considerable difference among any of the preparations. The ATP-sensitive potassium channel opener pinacidil significantly shortened repolarization in all types of tissue, although APD in Purkinje fibers was abbreviated to a larger extent than APD in endocardial, epicardial and M cells. Thus we can conclude that, similarly to our results concerning the inhibition of the slowly inactivating sodium current, the effect of $I_{K\text{-}ATP}$ activation with pinacidil was similar in epicardial, M and endocardial tissues, but much greater in Purkinje fibers.

3.5. Comparison of the electrophysiological effects of specific blockers of I_{Ks} andI_{Kr} in dog ventricular muscle and Purkinje fiber repolarization

Direct and detailed investigation of I_{Ks} - the slowly activating component of I_K - should be highly important since **1.** the relative small density of this current was supposed to be responsible for the longer APD in M cells (19), **2.** previous data obtained in the guinea pig suggested that compounds with selective I_{Ks} blocking properties have a more desirable profile of rate-dependent action and thereby better safety than currently available Class III compounds (18). Recently two compounds, chromanol 293B and L-735,821 were reported to block I_{Ks} with great specifity in guinea pig cardiac myocytes (22,23), but their actions on the cardiac action potential have not been studied in detail. Therefore we examined the cellular electrophysiological effects of these agents (10 μM/l and 100 nM/l, respectively), investigating the possible role of I_{Ks} in repolarization. A comparison with the role of I_{Kr} was also made using the specific I_{Kr} blockers d-sotalol (30 μM/l) and E-4031 (1 μM/l). The experiments were carried out by applying standard intracellular microelectrode technique and the whole-cell configuration of the patch-clamp technique in dog ventricular muscle and Purkinje fibers at 37 °C (37).

My colleague, Norbert Iost examined the effects of E-4031 and d-sotalol on I_{Kr}, as well as the effects of chromanol 293B and L-735,821 on I_{Ks} in isolated dog ventricular myocytes. It was found that E-4031 (1 μM/l) completely abolished and d-sotalol (30 μM/l) attenuated I_{Kr} tail currents. Similarly, chromanol 293B (10 μM/l) greatly reduced and L-735,821 (100 nM/l) completely abolished I_{Ks} (37). The next step was to study the effects exerted by equipotent concentrations of chromanol 293B and L-735,821 that blocked I_{Ks} on dog ventricular muscle and Purkinje fiber action potential configuration (Fig. 11) and to compare them with those of d-sotalol and E-4031 that blocked I_{Kr} (Fig. 12) at a BCL of both 1000 ms and over a wide range of stimulation cycle lengths (300 - 5000 ms) as well (Fig. 13).

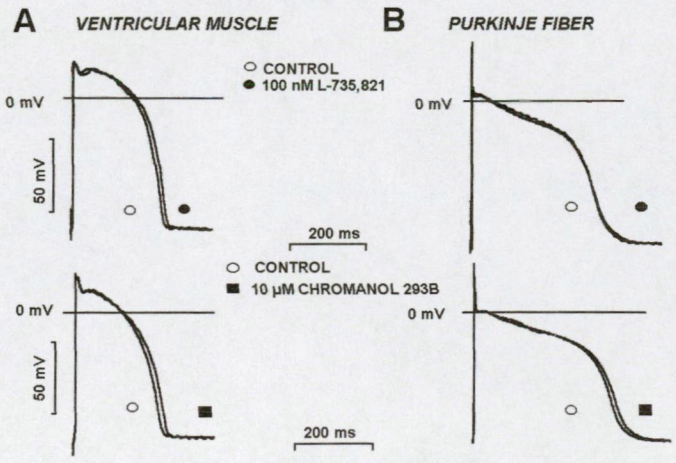


Figure 11. Action potential recordings from dog right ventricular papillary muscles **(Panel A)** and Purkinje fiber strands **(Panel B)** before and after 40 minutes superfusion with 100 nM/l L-735,821 (top) or 10 μM/l chromanol 293B (bottom) at BCL of 1000 ms.

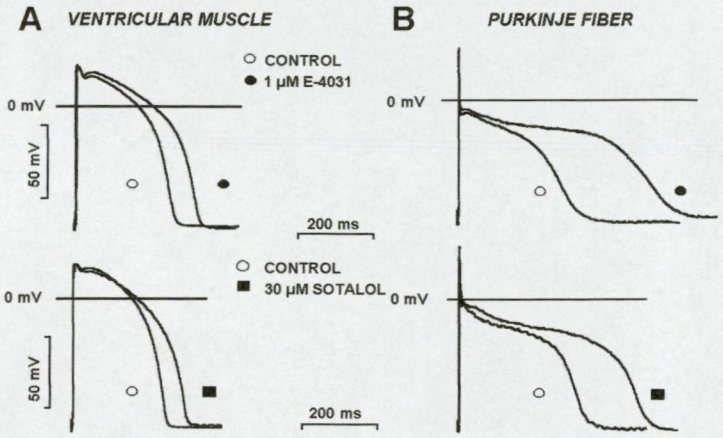


Figure 12. Action potential recordings from dog right ventricular papillary muscles **(Panel A)** and Purkinje fiber strands **(Panel B)** before and after 40 minutes superfusion with 1 μM/l E-4031 (top) or 30 μM/l d-sotalol 293B (bottom) at BCL of 1000 ms.

Chromanol 293B and L-735,821 produced small changes in APD amounting to less than a 7% increase over baseline measurements, and these unremarkable effects of I_{Ks} demonstrated little frequency dependence in both ventricular muscle and Purkinje fiber strands (Fig. 13.). In contrast, d-sotalol and E-4031 markedly lengthened both dog papillary muscle and Purkinje fiber APD (Fig. 12.) In addition, the increase in APD following I_{Kr} block occurred in a reverse frequency-dependent fashion so that the increase in APD was always greater at long cycle lengths than at short ones (Fig. 13.).

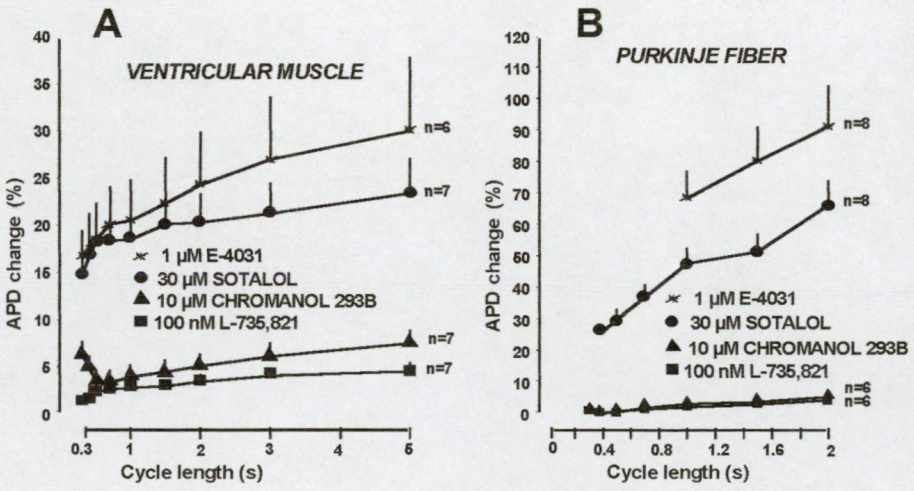


Figure. 13. Rate dependent effect of I_{Kr} (by E-4031 or d-sotalol), and I_{Ks} block (by chromanol 293B or L-735821) on APD in dog ventricular muscles **(Panel A)** and Purkinje fibers **(Panel B)**. Values are shown as mean ± SEM.

These results clearly show that I_{Kr} block lengthens APD greatly, while selective I_{Ks} block in dog has little effect on normal cardiac APD in both ventricular muscle and Purkinje fibers.

Since I_{Ks} is modulated by changes in intracellular cAMP, we also examined the effects of I_{Ks} block on APD in the presence of 1 μM/l forskolin to activate adenylcyclase and increase intracellular cAMP (Fig. 14). Forskolin alone (n = 18) markedly shortened APD in dog right

ventricular papillary muscle at BCLs ranging between 300 and 5000 ms (i.e. from 190.2 ± 4.4 ms to 157.1 ± 3.3 ms and 258.2 ± 5.7 ms to 212.5 ± 4.2 ms, respectively, at BCLs of 300 and 5000 ms). Addition of L-735,821 (100 nM/l) or chromanol 293B (10 μM/l) in the continuous presence of forskolin had little effect on APD (150.2 ± 2.2 ms versus 153.2 ± 2.6 ms and 207.5 ± 3.4 ms versus 209.0 ± 4.5 ms following L-735,821 and 164.1 ± 4.3 ms versus 176.0 ± 4.2 ms and 217.6 ± 5.1 ms versus 234.9 ± 9.1 ms following chromanol 293B at BCLs of 300 and 5000 ms, respectively). These results show again that selective I_{Ks} block only slightly lengthened APD over a wide range of stimulation frequencies, even in the presence of elevated intracellular cAMP.

Based on the data of the experiments carried out by our team (37) that measured the amplitudes and activation and deactivation time constants of I_{Kr} and I_{Ks} tail currents, the magnitude of I_{Ks} and I_{Kr} activated during the cardiac action potential were assessed, trying to to demonstrate the ratio of contribution of these two currents to the repolarization phase of the action potential in the dog ventricle (37). In agreement with earlier results (38), also in our study I_{Kr} activated rapidly during action potentials but deactivated slowly, while I_{Ks} activated slowly at more positive potentials. In addition, I_{Ks} accumulation over successive depolarisation is not likely since its deactivation is fast with respect to diastolic intervals occurring at physiological heart rates.

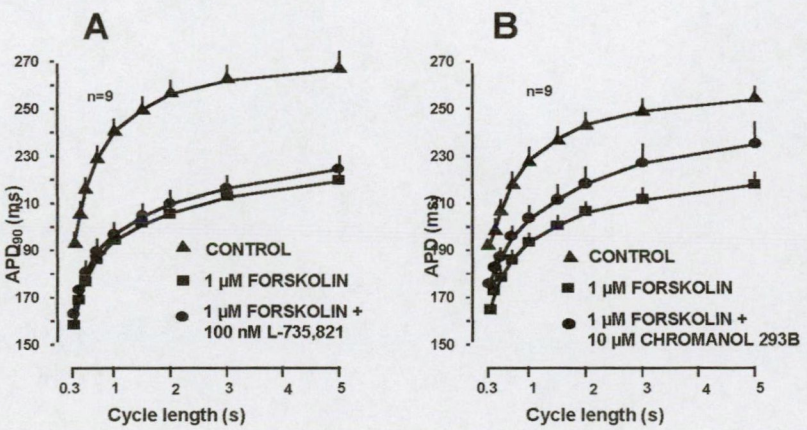
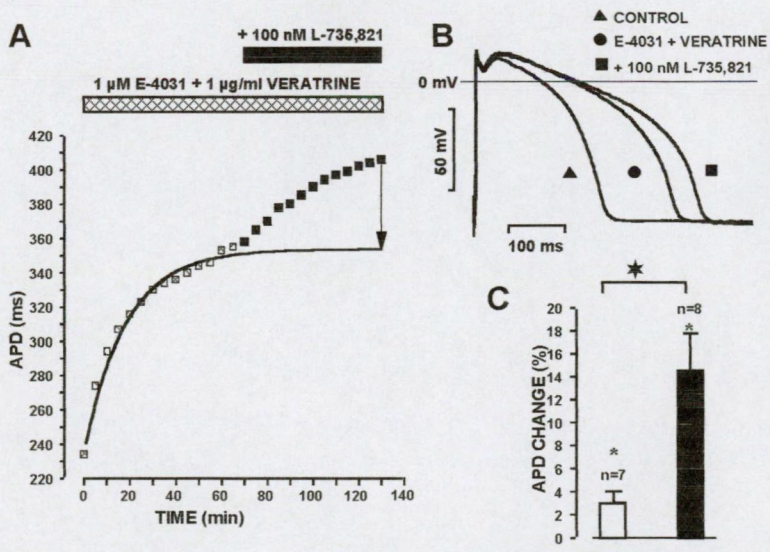


Figure 14. Frequency dependent effect of the I_{Ks} block by 100 nM/l L-735,821 **(Panel A)** or 10 μM/l chromanol 293B **(Panel B)** in the presence of 1 μM/l forskolin on APD in canine ventricular papillary muscles. Values are shown as ± SEM.

I_{Kr} and I_{Ks} kinetics such as these may account for the small effect of chromanol 293B and L-735,821 on APD at concentrations that completely or markedly blocked I_{Ks} in the present study. Furthermore, currents measured during and after 200 ms rectangular and artificial action-potential-like test pulses indicated that the outward current carried through I_{Kr} channels during the action potential is more than 10 times greater than through I_{Ks} channels (37). These

results agree well with the failure to increase ventricular and Purkinje fiber APD by blocking I_{Ks}, while I_{Kr} block caused marked lengthening.

Because we found I_{Ks} to have little role in normal action potential repolarization, we also examined its possible role when APD was artificially increased. In these experiments we tested the effects of both L-735,821 and chromanol 293B in dog ventricular papillary muscle action potentials, lengthened pharmacologically by exposure to 1 μM/l E-4031 (to block I_{Kr}) and 1 μg/ml veratrine (a recognised sodium channel agonist). While pacing continuously with BCL of 1000 ms, recordings were taken in every 5 minutes after initiating superfusion with 1 μM/l E-4031 + 1 μg/ml veratrine until a "quasi" steady-state was attained (Fig. 15.). Then, in the continued presence of E-4031 and veratrine that pharmacologically lengthened APD, the effects of I_{Ks} block were examined by either applying 100 nM/l L-735,821 or 10 μM/l chromanol 293B. L-735,821 markedly lengthened APD under these conditions from 385.5 ± 25.2 ms to 442.1 ± 32.3 ($p < 0.01$, $n = 7$) (Fig. 15.). This effect was in sharp contrast to the negligible effect of L-735,821 on normal APD (Figs. 11., 13.). Comparable effects on APD were obtained with chromanol 293B in the continuous presence of E-4031 and veratrine (APD was 366.1 ± 13.1 ms before chromanol 293B versus 429.5 ± 23.5 ms after its addition, $p < 0.01$, $n = 8$). These results indicate that the effect of I_{Ks} block on APD is substantially increased when APD is abnormally lengthened. In accordance with these results, currents measurements showed that when the duration of the rectangular or action-potential-like test pulse was increased, I_{Ks} was more fully activated (37).



Firure 15. Effect of 100 nM/l L-735,821 on dog ventricular action potentials recorded in the presence of 1 μM/l E-4031 and 1 μg/ml veratrine. **Panel A.** The time course of a representative experiment. At 0 minute 1 μM/l E-4031 and 1 μg/ml veratrine were added and measurements were taken in every 5 minutes until a "quasi" steady state was achieved. Then 100 nM/l L-735,821 was added to the bath in the continuous presence of E-4031 and veratrine. The relation prior to addition of L-735,821 was fitted by the following equation ($Y = A + B \exp(-X/C)$) to estimate the time-dependent changes that would have occurred in the absence of the I_{Ks}

blocker (solid line) so that the magnitude of its effect at 140 min is indicated by the arrow. **Panel B.** Representative action potentials recorded at baseline (0 min), after exposure to E-4031 and veratrine alone (70 min), and following addition of L-735,821 (130 min). **Panel C.** Comparison of the effect of L-735,821 on "short" (open bars) and on "long" (filled bars) dog ventricular action potentials, respectively recorded in the absence or presence of E-4031 and veratrine. Small asterisks represent significant changes from baseline measurements (i.e., at 0 min). Bold asterisk represents significant changes between the bars ($p < 0.01$ in both cases). Means ± SEM are shown.

To further examine whether I_{Ks} block lengthens APD and increases QT interval, the effects of chromanol 293B (1 mg/kg *i.v.*) and d-sotalol (1 mg/kg *i.v.*) in anaesthetised dogs were also examined. In agreement with our *in vitro* observations, chromanol 293B did not significantly affect QTc interval, while d-sotalol markedly legthened it (37).

Our results indicate that in contrast to specific I_{Kr} bolckers, specific inhibitors of I_{Ks} do not lengthen APD in either type of cardiac tissues. However, when APD is abnormally lengthened by other means, the effect of selective I_{Ks} blockers on repolarization increases substantially resulting in a considerable prolongation of the ventricular muscle APD.

4. Discussion

4.1. Electrophysiological effects of different antiarrhythmic agents

One of the goals of the present study was to investigate the *in vitro* cellular electrophysiological mechanisms of various antiarrhythmic drugs that act by lengthening the cardiac repolarization. Examing the effect of almokalant, cibenzoline and chronic amiodarone treatment in isolated dog right ventricular trabecular muscle it was found that all three drugs prolonged significantly ERP at 1 Hz stimulation frequency, which effect may be expected to be favourable in the management of ventricular reentry-type tachycardia. As for the impulse conduction time, the three agents proved to affect it in different manners. Almokalant did not change it at any of the stimulation frequencies applied and this is in concordance with earlier findings (39, 40) and indicates that this agent has no influence on the sodium channels. Amiodarone and cibenzoline increased conduction time in a rate-dependent fashion, suggesting the use-dependent inhibition of the sodium channels. Figures 1/A and B show, however, that while amiodarone depressed impulse conduction significantly only at high stimulation frequencies, i.e. this effect is only evident during tachycardia and at early extrasystoles, without

affecting conduction at normal heart rate, cibenzoline acted to lengthen conduction time at both slow, physiological and fast stimulation frequencies. This effect seems to be undesirable from the therapeutic point of view, since the depression of impulse conduction at normal heart rate can increase the occurance of proarrhythmia. On the basis of the results of our experiments it can be establish that cibenzoline markedly reduced the ERP shortening effect of cromakalim (Fig. 2.), suggesting the inhibition of the ATP-sensitive potassium channels that play an important role during cardiac ischaemia. It is now generally accepted that $I_{K\text{-}ATP}$ is responsible for the action potential shortening that occurs in heart muscle during metabolic blockade (41). These channels were characterized by a pronounced blockade of channel activity when millimolar concentration of ATP is present in the cell and they open when the cytosolic ATP level decreases below a critical threshold, resulting in the efflux of intracellular K^+ and in the increase of extracellular K^+ concentration. These changes induce the shortening of APD of myocytes in the ischaemic area and they also inhibit the speed of impulse conduction. These in turn can set the stage for reentry-type arrhythmias. In our experiments cibenzoline inhibited the ATP-sensitive potassium channels activated by cromakalim and this finding is in agreement with the literature (42), suggesting that therapeutic concentration of cibenzoline, that can be characterized as a Class IA drug, is effective in attenuating both intracellular potassium escape and the proarrhythmic, APD abbreviating effect of ishemia (43). It is generally accepted that Class IA drugs block also I_K (43), thus the question may arise as to whether the inhibition of cromakalim-induced ERP shortening caused by cibenzoline occurred, at least in part, via the blockade of I_K. This possibility, however, is not probable since almokalant that is a specific blocker of I_K failed to influence the ERP shortening effect of cromakalim. The theapeutical role of the pharmacological modification of ATP-sensitive potassium channels remains to be clarified. On one hand, their inhibition can be advantageous in the protection against reentry-type arrhythmias, on the other hand however several evidences proved that the opening of these channels and as a consequence the shortening of the action potential can serve as an important protective mechanism for the ischaemic myocardium. This is because the shorter the APD, the less Ca^{++} can enter the cell and this results in a decrease in contraction which is the major mechanism consuming ATP. Therefore, activation of $I_{K\text{-}ATP}$ prevents further depletion of ATP and protects the cell from irreversible impairment of its energy metabolism (41). In the light of the above it can be concluded that to decide wether the inhibitiory effect of cibenzoline on the cromakalim-induced ERP shortening

should be considered as a favourable antiarrhythmic mechanism or rather as an undesirable action that predispose to further injuries, needs further investigations.

Our findings are in agreement with the literature (13,39,42), and indicate that there are important differences between antiarrhythmic drugs known to prolong repolarization. These differences may have important implication during treatment of cardiac arrhythmias with the above agents.

The failure of the long-term antiarrhythmic therapy with Class IC and III agents shifted the interest toward amiodarone, a drug which seems to reduce postinfarction mortality (44). Amiodarone is a widely used old antiarrhythmic drug. Singh and Vaughan-Williams observed first the lengthening of APD after chronic treatment with the drug for 6 weeks in the rabbit (45). Since this discovery amiodarone has been termed a Class III antiarrhythmic drug. Later it was found that under acute conditions the drug use-dependently blocked V_{max} and the inward sodium current (29,46). It was also reported that amiodarone suppressed the inward calcium current (47). It is now clear that amiodarone is not a "pure" Class III drug, but it has multiple actions including blockade of the α- and β-adrenoreceptors (48). The most characteristic feature of the electrophysiological effects of amiodarone is that the action of the drug differs markedly after acute and chronic administration (49). On the basis of earlier experiments it can be expected that the cardiac electrophysiological effects of chronic amiodarone treatment are, at least partly, due to the formation and accumulation of an active metabolite, N-desethylamiodarone in the plasma and tissues (27). However, the mechanism of the effect of chronic amiodarone treatment seems to be more complex. Singh and Vaughan-Williams , as early as in 1970, observed the effect of long-term amiodarone treatment on thyroid function (45). Later it was demonstrated that experimental hypothyreodism attenuated the repolarization lengthening effect of chronic amiodarone treatment (50). These results suggest that the effects of long-term amiodarone treatment, as well as some of the serious side-effects accompanying it, are mediated, at least partly, via thyroid action (51). In this study the effects of the active metabolite, N-desethylamiodarone and a novel derivative of amiodarone lacking the iodine substituents, dronedarone, were characterized and compared with those of amiodarone in dog papillary muscles and Purkinje fibers.

The findings were as follows: (A) In Purkinje fibers, 10 μM/l desethylamiodarone produced significant shortening in both APD_{50} and APD_{90} at BCL of 1000 ms, and it also shortened

APD_{90} at different constant cycle lengths and at abrupt changes in stimulation cycle length as well. (B) Also in Purkinje fibers, the drug induced use-dependent depression of V_{max} with a recovery time constant of $\tau = 341.85$ ms that appears to be similar to that of amiodarone. (C) In ventricular muscle the drug exerted moderate use-dependent depression of V_{max} without changing the APD and other electrophysiological parameters. (D) 10 μM/l dronedarone, the recently developed iodine-free amiodarone analog, was also found to abbreviate significantly APD in Purkinje fibers, but not in ventricular muscle. (E) Similarly to desethylamiodarone, also dronedarone exerted use-dependent block of V_{max}.

It is well-known that APD in Purkinje fibers is normally longer than that in ventricular muscle. Therefore, the observed differential effect of both desethylamiodarone, dronedarone and amiodarone on the changes in APD (i.e. a marked shortening of the Purkinje fiber APD accompanied by no change of the APD in ventricular muscle) may contribute to an overall electrical stability in the heart and may possibly constitute an antiarrhythmic mechanism for the acute termination of cardiac arrhythmias.

The use-dependent effect on V_{max} (i.e. the largest depressant effect occurred at the shortest stimulation cycle length applied) found with both compounds as well as with amiodarone is only evident in depolarized tissue, and at high stimulation frequency (during tachycardia) or at early extrasystoles, without significantly affecting conduction of the action potentials at normal heart rate (Class I/B effect) (29). These features might be responsible not only for the antiarrhythmic efficacy, but also for the relative safety of these drugs in clinical settings.

In conclusion, our results suggest that the electrophysiological effects of both desethylamiodarone and dronedarone are different in cardiac Purkinje and ventricular muscle fibers, and similar to those observed with the parent compound amiodarone. Furthermore, it was found that despite its lack of iodinated substituents, dronedarone exerts similar acut electrophysiological effects to those of amiodarone, and this finding could account for its promising role in the treatment of ventricular arrhythmias.

Ambasilide is a new investigational antiarrhythmic agent, recently synthesized and being examined predominantly for Class III electrophysiological properties, however multiple pharmacological actions of the drug are also presumed (32,33,34), making ambasilide to seem to share striking similarities with amiodarone in several respects. That amiodarone is uniquely effective in a wide variety of arrhythmias and shows very little propensity to produce TdP,

provides the basis for investigating the *in vitro* rate-dependent electrophysiological properties of 10 and 20 μM/l ambasilide in canine Purkinje and ventricular muscle fibers. Our findings showed that the main effect of ambasilide is to lengthen APD_{90}, (a Class III antiarrhythmic action) in both types of tissues and in both concentrations that have no effect on the V_{max} when the fibers are stimulated at CLs longer than 700 ms (Table 1., Fig 3.). However, the drug exerted a differential effect on APD_{50} in the two types of preparations: in Purkinje fibers ambasilide shortened the phase 2 of the action potential while it prolonged phase 3, whereas both phases were prolonged in ventricular muscle. Several studies have indicated that the marked prolongation of APD_{50} (phase 2) in Purkinje fibers at long CLs might contribute to the development of EADs and triggered activity, and thus sets the stage for a variety of reentrant arrhythmias, including torsade de pointes (52). The effects of ambasilide proved to be different from those of pure Class III agents: the lack of a significant lengthening (in 10 μM/L) or even a decrease of APD (in 20 μM/L) in the plateau phase of the Purkinje fibers were observed (Table 1., Fig. 3.), which is probably due to the inhibitory effect of the drug on the slowly inactivating (53) or "window" sodium current (54), and as a consequence may reduce the probability for the development of EADs and triggered activity. There was also a difference in the effects on APD_{90} in Purkinje fibers versus those in ventricular muscle when the stimulation frequency was changed. Most drugs with Class III action prolong APD more at slower rates, producing little or no change at fast rates. This phenomenon, termed reverse use-dependence (55), is particularly evident in M cells and Purkinje fibers due to the preferential response of these cell types to agents that prolong APD (15). This feature of most Class III drugs can seriously limit their antiarrhythmic efficacy by compromising their ability to prolong APD and refractoriness when most needed, i.e. during tachyarrhythmia. In addition, it also contributes importantly to the proarrhythmia caused by most Class III agents because the dramatic prolongation of M cells and Purkinje fibers at slow rates leads to a marked increase in the TDR, setting the stage for a variety of reentrant arrhythmias (56,57). Our data indicate that in ventricular muscle both concentrations of ambasilide produced a fairly similar prolongation of the APD_{90} at all rates and therefore did not display reverse use-dependence (Fig. 4.). Although in 10 μM/l the APD prolonging effect of the drug proved to be clearly reverse use-dependent in Purkinje fibers, this effect was moderate after applying the higher concentration of ambasilide (Fig. 4.). Based on this observations, it can be expected that ambasilide would be less proarrhythmic than both the conventional and the recently developed, newer pure Class III

compounds. In this context it would also be of interest, however, to evaluate the effect of the drug also on M cells. Due to the inhibitory effect of the drug on both I_{Kr} and I_{Na}, it is likely that an increase in the concentration can induce a shortening of the APD of M cells, as does amiodarone in Purkinje fibers (26), resulting in a decrease of the TDR.

These findings of the present study in ventricular muscle are consistent with the results of former studies in guinea pig (58), dog (32) and human ventricular muscle (59) that have also shown the lack of reverse use-dependence in the action potential prolonging effect of the drug. This feature of ambasilide could be of particular importance in the development of antiarrhythmic therapy and was suggested to be due to the block of I_{Kr} and other repolarizing currents such as I_{Ks} (58), and, in atrial tissue I_{to} (transient outward current) and I_{so} (sustained outward current) (33,34).

The effect of ambasilide on the rate-dependent depression of V_{max} was also studied. Although even the higher concentration of the drug failed to produce a significant change in V_{max} at BCL of 1000 ms (Table 1.), thereby suggesting no inhibitory action on fast sodium channels at normal heart rate, at higher stimulation frequencies, however, a marked frequency-dependent depression of V_{max} was observed in ventricular muscle preparations after the application of both concentrations (Fig. 5/A). The use-dependent effect on V_{max} suggests depression of the inactivated fast sodium channels, and correspond with Class I/B effect (60). The findings about the use-dependent effect on V_{max} are in accord with the results of Takanaka et al. (32), who also demonstrated that ambasilide exerts inhibitory effect on the fast sodium channels at fast stimulation frequencies in dogs.

Examining the effect of 20 μM/l ambasilide on the recovery charasteristics of V_{max} in ventricular muscle, we indeed found that ambasilide depressed V_{max} at diastolic intervals shorter than about 1 s, (the recovery time constant of the drug was calculated to be $\tau = 1082.5 \pm 205.1$ ms, $n = 6$). This value appears to be close to the time constant of Class I/B agents (61), and definitely faster than that of Class I/A and Class I/C compounds (60). The onset kinetics of V_{max} block induced by ambasilide in this study was found to be intermediate between that reported of Class 1/B and Class 1/C antiarrhythmic drugs (35,61) and similar to that repoted of dispyramide (61). As far as we know, this is the first study demonstrating the effect of ambasilide on the recovery and onset kinetics of V_{max} block (Fig. 6.), which may serve as a basis for its classification.

Besides depressing V_{max}, ambasilide also produced a marked increase in the conduction time

(CT). This refers to the deceleration in the speed of impulse propagation, which effect is also related, at least partly, to the blockade of the fast sodium channels. Similarly to the inhibition of V_{max}, this action of ambasilide was also manifested at fast rates, ie. at short CLs (Fig. 5/B). On the basis of these findings, the drug can be characterized by relatively rapid offset kinetics from the fast sodium channels, a property which can be considered probably less proarrhythmic than the I/C type V_{max} depression caused by flecainide and encainide, i.e. drugs involved in the CAST study (6).

In this context it is noteworthy to summarize the similarities found between ambasilide and amiodarone in several aspects. 1. Despite inducing considerable prolongation in the time for ventricular repolarization amiodarone produces a low incidence of torsade de pointes, a feature that might be attributed to its frequency-independent lengthening effect on APD. In this regard our findings showed, in consistence with previous studies (32,62), that at least in the canine ventricular muscle, ambasilide does not exhibit reverse use-dependence concerning repolarization. 2. The action of ambasilide resembles the effect of treatment with amiodarone, insofar as it either abbreviates phase 2 of the Purkinje fiber action potential (27) or produces markedly less prolongation of APD_{50} in Purkinje fibers than in ventricular muscle (25), the consequence of which is a reduction in the probability for development of EADs and triggered activity. 3. Both ambasilide and amiodarone possess important Class I/B type sodium channel blocking activity (29), especially under conditions in which sodium channels are partially inactivated. In all these respects, amiodarone and ambasilide differ markedly from other conventional and newer Class III antiarrhytmic agents and neither of them fit well into the conventional antiarrhythmic classification scheme (63). The similarity found in all the above actions of ambasilide and those of amiodarone suggests that because of its advantageous, "multifaced" electrophysiological profile, ambasilide may be a promising drug candidate for antiarrhythmic drug therapy.

4.2. Electrophysiological and pharmacological heterogeneity within the ventricle: the role of M cells

In this study the cellular electrophysiological and pharmacological properties of M, endocardial, epicardial and Purkinje cells of the dog left ventricle were examined and compared. We confirmed the existence of M cells in the canine left ventricle described earlier

(16). In addition, we also revealed important differences in certain action potential parameters of M cells, which were not previously mentioned by others. It was also demonstrated in the present study that M cells differ from Purkinje fibers in some aspects which were not obvious from previous investigations; 1. the early portion of the APD restitution curve in M cells is more similar to that of endocardial and epicardial cells than to Purkinje fibers (Fig. 9.), 2. the potential range of the plateau phase in the M cell action potential is also more similar to that of endocardial and epicardial cells than to Purkinje fibers (Fig. 7.), 3. the pharmacologic response of M cells to TTX or pinacidil resembles more the endocardial and epicardial cells than the Purkinje fibers (Fig. 10.).

In recent years a growing number of studies have stressed the importance of diversity within the ventricles of the heart attaching great significance to differences in the electrophysiological characteristics and pharmacological responsiveness of M cells located in the deep structures of canine (16,19), guinea pig (64), rabbit (65), and human ventricles (66). These cells are distinguished mainly by the ability of their action potential to prolong disproportionately to the other cell types with slowing of the stimulation rate and their greater sensitivity to agents and interventions that prolong APD. Besides the marked rate-dependent change of APD in M cells, the high V_{max} value of their action potential resembles also those observed in Purkinje fibers (16). However, M cells show no phase 4 depolarization, not even in the presence of catecholamines and low potassium concentration. Therefore it was concluded that M cells display characteristics common to both myocardial cells (spike and dome morphology, absence of phase 4 depolarization) and Purkinje fibers (higher V_{max}, steeper APD-rate relation). Our findings further delineate the distinctions among cells spanning the wall of the canine left ventricle.

Action potentials recorded in the four tissues of the canine ventricle exhibit marked differences in morphology (Fig. 7.). Prominent among these is the presence of an expressed phase 1 in epicardial, M and Purkinje cells but less in endocardial fibers. Aside from differences in the early phases of the action potential, a significant distinction exist among the four cell types with respect to phase 3 repolarization, the result of which is a progressive prolongation of APD in M cells and Purkinje fibers relative to epicardial and endocardial cells with the slowing of rate. Also, Purkinje fibers displayed plateau phase at a more negative potential range than the other cell types studied. Our data concerning action potential characteristics are in agreement with those of Sicouri & Antzelevitch (16), who described similar results previously. In addition, we

also could reveal important differences in certain action potential parameters of M cells which were not described earlier by others, namely the distribution of V_{max} and action potential amplitude values reflecting probably the inhomogeneity of the fast sodium current in these cells (Table 3.). It has to be noted that action potentials recorded in the two groups of cells displayed characteristics of M cells, i.e. they possessed the ability to prolong disproportionately APD when compared with epicardial and endocardial fibers in response to a slowing of the stimulation rate. To our knowledge, the present study is the first to demonstrate that concerning the magnitude of V_{max}, which probably reflects the intensity of the fast sodium current, M cells are not uniform.

The rate dependence of APD is the major feature differentiating M cells from the other two cell types present within the ventricular wall and making them more similar to Purkinje fibers. Examing the steady-state rate-dependence of APD in the four types of tissue we found, in concordance with the literature (16), that the slope of APD-cycle length relation was clearly much steeper for M cells than that for epicardial and endocardial cells at all rates of stimulation except for BCLs shorter than 500 ms, while it was remarkably similar to that observed in Purkinje fibers, which displayed an even more pronounced APD rate relation than M cells. There were no significant differences in steady-state APDs at all stimulation rates between endocardial and epicardial cell preparations.

The greater prolongation of the M cell response could give rise to a prominent dispersion of repolarization and refractoriness between the cells in the M region and cells in other parts of ventricular myocardium, as well as a dispersion of repolarization between the myocardium and the His-Purkinje system (67) as stimulation rate is slowed. This heterogeneity provides an important substrate for a variety of reentrant arrhythmias, including intramural reentry and TdP, and regarding that M cells are estimated to constitute at least 40% of the total ventricular myocardial mass (68), their possible role in arrhythmogenesis is especially of great significance. Based on the available data on TDR within the ventricle, Antzelevitch suggested (69) that since M cells may be the most abundant cell population in the ventricles and may represent the true working myocardial cells of these cardiac chambers, they may have evolved for the purpose of improved pump efficacy especially at slow rates at which more enduring depolarizations permit longer and more efficient contractions. Epicardium and endocardium may have developed to prevent dramatic prolongation of the M cell action potential and the development of afterdepolarizations. Accordingly, removal of a section or infarction of a

segment of epicardium or endocardium would be expected to lead to an increase of the QT interval and QT dispersion secondary to a prolongation of the M cell APD (70,71). In patients treated with drugs exhibiting Class III antiarrhythmic actions or in those with the congenital or acquired long QT syndrome, these effects of infarction to transiently increase QT and QT dispersion might be even more amplified (56). The implication of this might be an arrhythmic substrate capable of maintaining both monomorphic and polymorphic arrhythmias.

The remarkable distinction between the restitution of APD in M cells and Purkinje fibers (Figs 8.,9.) also represent an important electrophysiological difference between the two types of fiber, which has not been described yet. In order to explore the ionic nature of the differences in the restitution of APD among the four ventricular tissue types, however, further research is required, with more direct experimental methods like the patch-clamp technique.

It has been already reported that the four distinct cell types of the ventricles show different - in some cases opposite - responses to a wide variety of pharmacological agents (14,26,72,73,74, 75,76,77,78). Although the electrophysiological actions of sodium and calcium channel block and the activation of ATP-sensitive potassium channels have been well characterized in Purkinje and endocardial preparations (54,79,41), *in vitro* investigations of the same interventions are limited in epicardial (14,72,73,79) and especially in M cells (80). Therefore, we compared the pharmacological response of the four tissue types to the sodium channel blocker tetrodotoxin, calcium channel blocker nifedipine, and ATP-sensitive potassium channel activator pinacidil. Our results show that TTX shortened APD in both M cells and Purkinje fibers significantly, whereas no significant changes in APD were observed in epicardium or endocardium after application of this agent. Despite the qualitatively similar effect of TTX on M cells and Purkinje fibers, it has to be emphasised that the degree of APD abbreviation was more accentuated in Purkinje fibers than in M cells, suggesting a weaker contribution of the sodium current to repolarization in M cells than in Purkinje fibers, findings making M cells to be more akin to epicardial and endocardial cells. The organic calcium channel blocker nifedipine was found to reduce APD in all the four tissue types significantly without displaying remarkable difference with respect to the extent of abbreviation in any of the preparations.

Our data about the effects of pinacidil that is known to augment $I_{K\text{-}ATP}$ in cardiac tissues (81) show that the relatively high concentration (10 μM/l) of the drug caused a significant abbreviation of repolarization in all the four tissue types. Nevertheless, APD was shortened more excessively in Purkinje fibers than in ventricular muscle fibers. Thus we can conclude that

the pharmacological response to tetrodotoxin and pinacidil in M cells resembles more that in the epicardial and endocardial cells than that in the Purkinje fibers (Fig. 10.).

In conclusion, our results provide further important evidence in support of the existence of M cells in the deep subepicardium of the canine ventricle and indicate that there are significant electrophysiological as well as pharmacological differences between M cells and Purkinje cells. The establishment of complete profiles of the pharmacological responsiveness of the four cell types in the ventricle may narrow the gap that currently exists in this area and should bring us a step closer to a more definitive, evidence-based and less empirical approach in the medical management of cardiac arrhythmias.

4.3. Pharmacological modulation of the delayed rectifier potassium current in dog ventricular muscle and Purkinje fibers

I_{Ks} and I_{Kr} are both generally accepted as having important roles during normal action potential repolarization (18,82). The assumed importance of I_{Ks} is also underscored by the fact that the relative small density of this current was proposed to be the main reason for the longer repolarization observed in M cells relative to epicardial and endocardial cells (19). It has been known for long that specific I_{Kr} blockers (e.g. d-sotalol, dofetilide, E-4031) greatly lengthen cardiac APD (21,83) and thus provide antiarrhythmic benefit by increasing refractory wave length. However, these drugs also increase risk for development of bradycardia-induced polymorphic ventricular tachyarrhythmias (84). The APD increase induced by selective I_{Kr} blockade displays reverse use-dependency (55) that is especially pronounced in Purkinje fibers (20). In contrast with I_{Kr} blockers, however, on the basis of previous data obtained in the guinea pig (21), selective I_{Ks} block has generally been assumed to increase APD and refractoriness in frequency-independent manner, possessing thereby better safety than currently available Class III compounds. Accordingly, development of such drugs are expected to offer favourable new options for antiarrhythmic drug therapy. However, selective I_{Ks} blockers have only recently been available (22,23).Recent studies using canine ventricular myocytes have revealed considerable species differences in the properties of I_K (38) and the important question therefore arise as to how findings obtained from the guinea pig may be extrapolated to other mammalian species. With the development of specific I_{Ks} blockers, it has become possible to directly determine the effect of I_{Ks} on APD, and thus our aim was to

establish the role of this current in producing normal cardiac action potential repolarization in dog by comparing the magnitude and extent of changes in the first instance in ventricular muscle and Purkinje fiber APD produced by selective block of I_{Kr} and I_{Ks}. Our presumption was that since I_{Ks} activation starts at around 0 mV (37) and this voltage is more positive than the normal Purkinje fiber action potential plateau voltage (Fig. 7.) (36), I_{Ks} block should not be expected to increase Purkinje fiber APD, but only that of ventricular muscle, due to the fact that in this latter type of cells the action potential plateau voltage is more positive ($\approx$ +20 mV), allowing I_{Ks} to be substantially more activated, and resulting in a marked increase of APD after the blockade of this current. On this basis specific I_{Ks} blockers may produce antiarrhythmic benefit by decreasing enhanced dispersion in repolarization between ventricular muscle and Purkinje fiber, and thus limiting arrhythmogenesis. Our results supported this assumption only in part, since in addition to Purkinje fiber also in papillary muscle preparations both chromanol 293B and L-735,821, purportedly selective I_{Ks} blockers, failed to markedly lengthen APD (Figs 11.,13.). Equivalent concentrations of both compounds, however, substantially blocked I_{Ks} in isolated dog ventricular myocytes (37). Adenylcylase stimulation by forskolin, known to increase I_{Ks} (85), did not considerably alter the chromanol 293B or L-735,821 induced change in APD in dog papillary muscle (Fig. 14.). In contrast, E-4031 and d-sotalol (recognized I_{Kr} blockers) markedly lengthened dog ventricular muscle and Purkinje fiber APD (Figs 12.,13.). In agreement with these *in vitro* results, QTc was increased *in vivo* by d-sotalol but not by chromanol 293B in anaesthetised dogs. However, in papillary muscle preparations where APD was prolonged by E-4031 and veratrine, both chromanol 293B and L-735,821 increased repolarization considerably (Fig. 15.).

The few published studies that have examined the effect of I_{Ks} on cardiac APD are rather contradictory. Bosch et al. (86) for example, using the whole-cell patch-clamp technique in single isolated guinea pig and human myocytes, reported that APD increased following chromanol 293B exposure. In that study, a relatively small number of cells (5-8 cells) were examined and measurements in the absence or presence of chromanol 293B were made in different myocyte groups. It is also notable that APD measurements in single myocytes inherently show enormous beat-to-beat variability that makes identification of the effects of selective ion channel block on action potential configuration uncertain, at best. Nevertheless, our results using standard intracellular microelectrode recordings in dog papillary muscles agree, in part, with those of Schreieck et al. (87) in guinea pig papillary muscle; i.e., 10 μM/l

chromanol 293B did not lengthen APD in the absence of forskolin. However, in dog papillary muscle, we found no increase in APD after adenylcyclase stimulation as Schreieck et al. (87) did, following isoproterenol exposure in guinea pig. This deviation from the findings of Schreieck et al. (87) might be due to species differences. Certainly I_{Ks} amplitude is relatively large in the guinea pig (18) compared to dog and other species (38). In addition, we applied 1 μM/l forskolin while Schreieck et al. (87) used 100 nM/l isoproterenol to activate adenylcyclase. Because other currents are also modulated by cAMP (e.g., I_{Ca} and I_{Cl}) that also affect APD (88), the observations in the two studies may not be directly due to I_{Ks} block. I_{Ks} block, in our study, produced substantially different effects in multicellular dog cardiac Purkinje fiber strands than in the paper of Cordeiro et al. (89), who reported marked APD lengthening in isolated rabbit cardiac Purkinje fiber cells after superfusion with only 20 nM/l L-735,821. The reason for this discrepancy in findings is unknown, although the action potential plateau voltage in the rabbit Purkinje fiber cells illustrated by Cordeiro et al. (89: Fig. 9.) is approximately -20 mV, while in the same study (89: Fig. 11.) these authors show that activation of the L-735,821 sensitive current (presumably I_{Ks}) occurs at voltages positive to 0 mV. These facts make it unlikely that the observed increase in APD reported by Cordeiro et al. (89) was due to I_{Ks} block. The effects of d-sotalol and E-4031 on Purkinje fiber APD in our study are in excellent agreement with those previously published (18,84).

Prior to our report (37), I_{Ks} was believed vital to control normal cardiac action potential repolarization (19,82). In addition, based on experiments performed in guinea pig ventricular myocytes, selective I_{Ks} block was believed to increase APD without producing undesired, reverse use-dependent APD lengthening characteristic of I_{Kr} block (21). This expectation was based on the findings that I_{Ks} deactivates slowly in guinea pig so that reduction in outward current due to its block would be expected to be greater at fast heart rates (short diastolic intervals) than at slow heart rates or long intervals between subsequent action potentials. More recently, however, both in dog ventricular myocytes (38) and human ventricular myocytes (90), I_{Kr} has been demonstrated to deactivate slowly while I_{Ks} deactivates relatively rapidly. This is quite unlike the situation in the guinea pig and brings the speculation originally presented by Jurkiewicz & Sanguinetti (21) into question. Our finding that I_{Ks} block does not remarkably increase APD in either normal dog ventricular muscle or Purkinje fibers over a wide range of stimulation frequencies directly contradicts the 1993 Jurkiewicz & Sanguinetti hypothesis, as well as the hypothesis of Liu & Antzelevitch (19) who showed in isolated dog

ventricular myocytes that M cells express a lower density of I_{Ks} channels than do subendocardial or subepicardial cells. On this basis these latter authors postulated that the longer M cell APD was due to less repolarizing current flowing through I_{Ks} channels. Our present data, however, indicate that an 80 - 100% I_{Ks} block failed to substantially lengthen APD in dog subendocardial papillary muscle; i.e. substantial I_{Ks} block did not cause subendocardial cells to resemble M cells. Thus, differences in other membrane currents, such as sodium window currents (or slowly inactivating I_{Na}) density for example (80), may help account for the differences in M cell and subendocardial ventricular muscle cell action potential configurations.

Although I_{Ks} may have little role in normal action potential repolarization, it likely plays a vital role when cardiac APD is abnormally lengthened by other mean (e.g., by reductions in I_{Kr} or I_{K1} or increases in I_{Na} or I_{Ca}). This speculation is supported by our experiments where APD was substantially increased by pharmacological means of augmenting inward (I_{Na}) and decreasing outward (I_{Kr}) currents (Fig. 15.) Also our estimation of I_{Kr} and I_{Ks}, when the duration of the rectangular or action-potential-like test pulse was increased, showed that I_{Ks} was more fully activated than during the normal action potential (37). Thus, I_{Ks} is expected to limit excessive APD lengthening when repolarization is abnormally lengthened. As such, pharmacological block of I_{Ks} might be expected to have severe detrimental consequences when this protective mechanism is eliminated. For example, if repolarization is excessively lengthened due to drug induced I_{Kr} block, hypokalaemia, genetic abnormality, or bradycardia, the subsequent increase in APD would favour I_{Ks} activation and provide a negative feedback mechanism to limit further APD lengthening. Without such a mechanism, excessive APD lengthening might lead to enhanced regional repolarization dispersion (57) and increase propensity for development of EAD associated with TdP induction. Such a role for I_{K} in limiting excessive APD lengthening was first postulated by Ito and Surawicz (91), and if I_{Ks} plays such a role, antiarrhythmic agents producing nonselective block of I_{Kr} and I_{Ks} (e.g., quinidine and azimilide) might be associated with a greater proarrhythmic risk than "pure" (selective) I_{Kr} blockers (e.g., sotalol and dofetilide).

In conclusion, this study indicates that in normal dog ventricular muscle I_{Ks} plays a minor role in control of APD. This current, however, could provide an important means of limiting excessive APD lengthening when action potentials are increased beyond normal by other means.

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7. Annex

Publications related to the subject of the Thesis