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A cibenzolin, az almokalant és az amiodaron elektrofiziológiai hatásainak összehasonlító vizsgálata kutyaszívből izolált jobb kamrai trabekuláris izmon

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SZENT-GYÖRGYI ALBERT ORVOSTUDOMÁNYI EGYETEM PHARMACOLÓGIAI INTÉZET
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Kulcsszavak: *repolarizáció • frekvenciafüggés • effektív refrakter periódus • ingerületvezetés • ATP-függő K-csatorna*

Összefoglalás: Extracelluláris elektrofiziológiai módszerrel vizsgáltuk a szívizomsejtek repolarizációját megnyújtó hatású három antiaritmiás szer, a cibenzolin (10 μM), almokalant (100 nM) és az amiodaron kezelés (25 mg/kg/nap, 4 hét + 5 μM a szervfürdőben) frekvenciafüggő (0,5-4 Hz ingerlési frekvencia) hatásait az ingerületvezetési időre és az effektív refrakter periódusra (ERP) kutyák szívének jobb kamrai trabekuláin. Az említett szereknek az ATP-függő káliumáramra kifejtett esetleges hatásait olyan preparátumokon vizsgáltuk, amelyeken az ATP-függő K-csatornákat előzetesen 2 μM cromakalim alkalmazásával aktiváltuk. Eredményeink arra utalnak, hogy a repolarizációt megnyújtó szerek hatásmechanizmusa egymástól eltérő lehet, amelynek hátterében feltehetőleg e szerek különböző káliumcsatorna gátló hatása áll. Mindez fontos lehet a szív ritmuszavarainak a vizsgált szerekkel végzett kezelése során.

Keywords: *repolarization • frequency dependence • effective refractory period • impulse conduction • ATP sensitive K-channel*

Summary: The aim of the study was to investigate the effects of three antiarrhythmic drugs (cibenzoline 10 μM, almokalant 100 nM and amiodarone treatment p.o. 25 mg/kg/day for 4 weeks + 5 μM amiodarone in the tissue bath) which delay repolarization in cardiac muscle, on impulse conduction and effective refractory period (ERP) at wide range of stimulation frequencies (0,5-4 Hz) in dog right ventricular trabecular muscle by applying extracellular electrophysiological technique. The possible effects of the drugs on the ATP-sensitive potassium current (IKATP) were tested in fibers where the IKATP channels were activated by 2 μM cromakalim. Our results suggest that there are distinct differences between antiarrhythmic drugs known to lengthen repolarization. These differences may have important implication during treatment of cardiac arrhythmias with the above agents.

Levelezési cím: DR. BALÁTI BEÁTA,
SZOTE Pharmacológiai Intézet Szeged 6701, Pf. 115.

Kísérleteinkben három, a szívizom repolarizációját megnyújtó antiaritmiás szer, az almokalant, a cibenzolin és az amiodaron szívelektrofiziológiai hatásait hasonlítottuk össze. A Vaughan Williams féle klasszifikáció (17) szerint, az almokalant „tisztán" a III. osztályba tartozik, tehát szelektíven csak a repolarizációt megnyújtó hatással rendelkezik (2), a cibenzolin a repolarizáció megnyújtása mellett az ingerületvezetést is gátolja, így az I/A osztályba sorolható (8), míg az amiodaron ezen klasszifikáció mind a négy osztályára jellemző elektrofiziológiai hatásokkal rendelkező, egyedülállóan komplex vegyület (10, 14). A szívizomsejtek repolarizációját megnyújtó szerek fontossága a CAST vizsgálat (Cardiac Arrhythmia Suppression Trial) eredményeinek ismertté válása óta (1989.) megnőtt (12, 14), és azóta a kamrai tachycardiák kezelésében is a hangsúly egyre inkább a III. osztályba tartozó, repolarizációt megnyújtó hatású szerekre helyeződött át, elérően a jelenleg használt antiaritmiás gyógyszerkincsünk zömét kitevő I. osztályba sorolt szerektől. A K-csatorna gátló gyógyszerek (III. osztály) antiaritmiás hatása elsősorban a pitvari és kamrai myocardium refrakter periódusának megnyújtása révén érvényesül, s ebből következően ezek a „reentry"-n alapuló supraventriculáris és kamrai tachycardiák megszűntetésének és megelőzésének hatékony gyógyszerei.

Jelen tanulmányunkban három, a repolarizációt megnyújtó antiaritmiás szer, a cibenzolin, az almokalant és az amiodaron szívelektrofiziológiai hatásait vizsgáltuk és hasonlítottuk össze.

KÍSÉRLETI MÓDSZER

Kísérleteinkben iv. 30 mg/kg pentobarbitállal túlaltatott, 8–12 kg súlyú, vegyesnemű korcs kutyák szívéből kipreparáltuk a jobb kamrai trabekulák felszíni rétegeit. A preparátumokat Krebs oldattal (128,0 mM/l NaCl, 21,4 mM/l $NaHCO_3$, 10,01 mM/l glukóz, 4,0 mM/l KCl, 1,8 mM/l $CaCl_2$, 0,42

mM/l $MgCl_2$) átáramoltatott szervfürdőben rögzítettük. A tápoldatot folyamatosan karbogén gázzal (95% O_2 és 5% CO_2) buborékoltattuk át. A hőmérséklet a kísérleti kádban 37±0,5 °C, a pH 7,3±0,5 volt. A preparátumokat 2 ms időtartamú kétszeres ingerküszöbű, 1 Hz frekvenciájú (= 1000 ms ciklushosszú) impulzusokkal, bipoláris platina elektródok közvetítésével ingereltük (EMG 4767). Az ingerületet a készítmény felszínére helyezett bipoláris platina elektróddal vezettük el és erősítőrendszeren (Eltron biológiai erősítő) keresztül oszcilloszkópba vezettük (EMG RT4657), melyen a jel regisztrálása történt.

A vizsgált paraméterek az ingerületvezetési idő és az effektív refrakter periódus (ERP) voltak. Ismert, hogy az antiaritmiás szerek szívizomsejtekre kifejtett hatása frekvenciafüggő (13). Ezzel összhangban a cibenzolin, az almokalant és az amiodaron frekvenciafüggő hatásait vizsgáltuk az előbb említett két paraméterre.

Az ingerületvezetési időt az ingerlő és az elvezető elektród között haladó jel terjedési idejével jellemeztük.

A méréseket 60 perc beállítási idő előzte meg, amikor a készítményeket 1 Hz frekvenciával ingereltük. A beállási idő után először egy kontroll méréssorozatot végeztünk, melynek során az ingerlési frekvenciát 0,5 és 4 Hz között változtattuk. Az új ingerlési frekvenciára történt átállás és a mérés között két perc adaptációs szünetet tartottunk. Az ingerküszöböt minden új ciklushossznál úgy mértük, hogy az inger intenzitását fokozatosan csökkentettük a kétszeres küszöbértékről egészen addig, amíg a tovaterjedő akciós potenciálokat már nem lehetett kiváltani. Ezt követően automata pipettával a szervfürdőhöz adtuk a megfelelő koncentrációjú vizsgált szert. A szükséges inkubációs idő után újabb méréssorozatot végeztünk. A cromakalim hatását 10 perces, a cibenzolinét 40 perces, az almokalantét 60 perces beállási időt után, az amiodaron (5 μM/l) hatását a krónikusan kezelt kutyákból származó preparátumokra pedig 60 és 120 perces inkubációs idő után mértük.

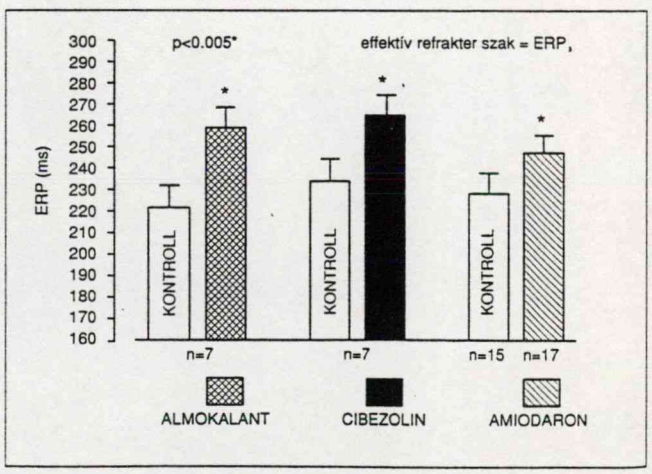
A szerek koncentrációja a cibenzolin (UPSA) esetében 10 μM/l az almokalantnál (ASTRA) 100 nM/l volt. A krónikus amiodaron kezelés során 10 állat kapott 4 héten keresztül napi 25 mg/kg amiodaront (Sanofi). A krónikus amiodaron kezelésben részesülő állatok EKG paramétereit naponta, bipoláris mellkasi elvezetésű EKG-val regisztráltuk és csak a szer QTc megnyúlást okozó hatásának (5, 14) észlelését követően került sor az izolált szervi kísérletek elvégzésére. Az amiodaronnal krónikusan előkezelt állatokból nyert preparátumok mérésénél a szervfürdőhöz is adtunk 5 μM/l amiodaront. Erre a szer a preparátumokból történő kimosódásának elkerülése miatt került sor, és így az amiodaron kísérletesen igazolt akut hatását (9) is vizsgálhattuk. A koncentrációk megválasztásánál a cél a terápiás viszonyok megközelítése volt.

Az ATP-függő K-áramra kifejtett esetleges hatásokat 2 μM/l cromakalim alkalmazásával vizsgáltuk. Ismert, hogy a cromakalim az ATP-függő K-csatornák megnyílását eredményezi és így az effektív refrakter periódust rövidíti. Kísérleteink során ATP-függő K-csatorna gátló hatásnak tekintettük azt, ha a vizsgált szer mérsékelte vagy kivédte a cromakalim okozta ERP rövidítést.

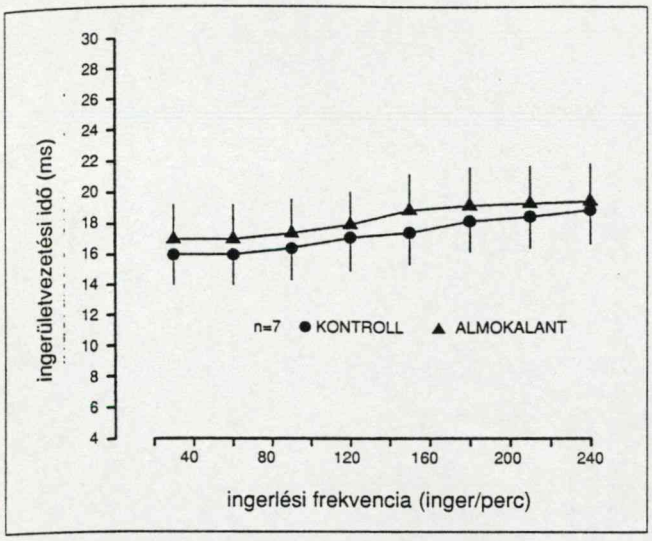
Az eredmények statisztikai értékelésénél a Student-féle páros t próbát alkalmaztuk. Az amiodaronnal krónikusan előkezelt állatokon végzett kísérletek értelemszerűen nem önkontrollosak voltak, hanem az almokalanttal és a cibenzolinnel végzett kísérletek kontroll adatait használtuk fel összehasonlításként. A kísérletek statisztikai kiértékelése ebben az esetben a páratlan t-próbával történt. A változásokat akkor tartottuk szignifikánsnak, ha a p értéke kisebb volt 0,05-nél.

EREDMÉNYEK

Az almokalant, cibenzolin és az amiodaron effektív refrakter periódusra kifejtett hatását az *1. ábra* szemlélteti. Az ábrából kitűnik, hogy 1 Hz ingerlési



1. ábra. *Az almokalant (100 nM), cibenzolin (10 μM) és az amiodaron (25 mg/kg/nap, 4 hét + 5 μM a szevfürdőben) hatása kamrai munkaizomrostok effektív refrakter periódusára 1 Hz ingerlési frekvenciánál. Az ordinátán az effektív refrakter periódus időtartamát tüntettük fel. Az ábrán a ± standard error és a szignifikancia szerepel.*



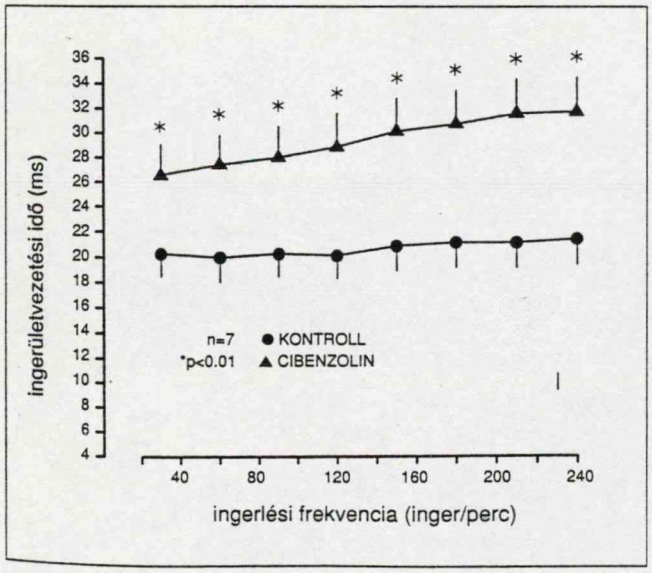
2. ábra. *Az almokalant (100 nM) frekvenciafüggő hatása az ingerületvezetési időre kamrai munkaizomrostokon. Az abszcisszán az ingerlési frekvenciát, az ordinátán az ingerületvezetési időt tüntettük fel. Az ábrán a ± standard error szerepel.*

frekvenciánál mindhárom vizsgált szer szignifikánsan megnyújtotta az effektív refrakter periódus időtartamát. A vizsgált szerek hatásait az ingerületvezetési időre a *2., 3. és 4. ábra* szemlélteti. Az almokalant az ingerületvezetési időt egyik ingerlési frekvenciánál sem változtatta meg szignifikáns mértékben (2. ábra). A cibenzolin már alacsony (0,5 Hz = 30 inger/perc) ingerlési frekvenciánál is szignifikánsan gátolta az ingerületvezetést (29,8 ±7,3%, n = 8, p<0,05) és ez a hatás a frekvencia növelésével tovább fokozódott (3,5 Hz = 49,4 ±9,3%, n = 8, p<0,05), (3. ábra).

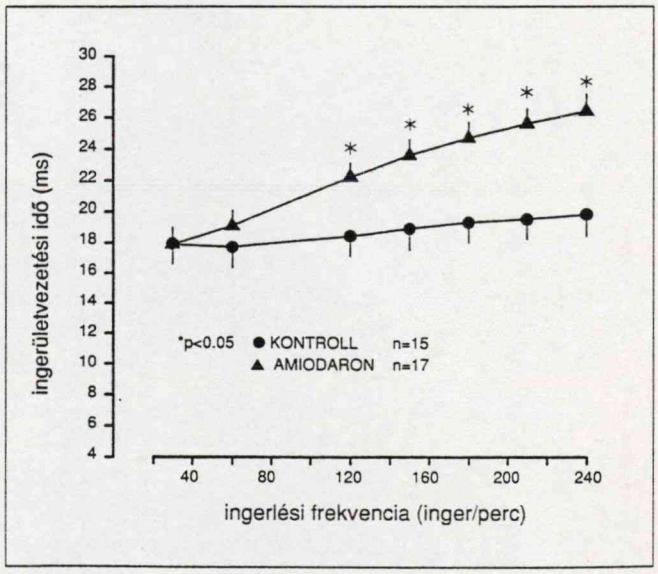
Az 5 μM/l amiodaron a krónikusan kezelt állatok szívén vizsgálva csak az 1 Hz-nél magasabb frekvenciájú ingerlésnél gátolta szignifikánsan az ingerületvezetést, amely hatás jelentősen erősödött az ingerlési frekvencia növelésével. Amint ez a 4. ábráról is látható, eltérően a cibenzolinnál tapasztaltól, az amiodaron 0,5 Hz-nél még nem gátolta szignifikánsan az ingerületvezetést, viszont 1 Hz-nél már a kontrollhoz képest 7,5 %-kal (n = 22, p< 0,05) megnyújtotta az ingerületvezetési időt. Ez a hatás a frekvencia növelésével tovább fokozódott (3,5 Hz = 33%, n = 22, p<0,05), (4. ábra).

A szerek ATP-függő K-áramra kifejtett esetleges hatásait 2 μM/l cromakalim alkalmazásával vizsgáltuk. A cromakalim, mint már említettük, az ATP-függő K-csatornák megnyitását eredményezi, így az ERP-t rövidíti. A cromakalim ERP rövidítő hatásának kivédéséből illetve mérsékléséből következtettünk az ATP-függő K-csatornák gátlására. Ezen kísérlet eredményét az 5. ábra mutatja.

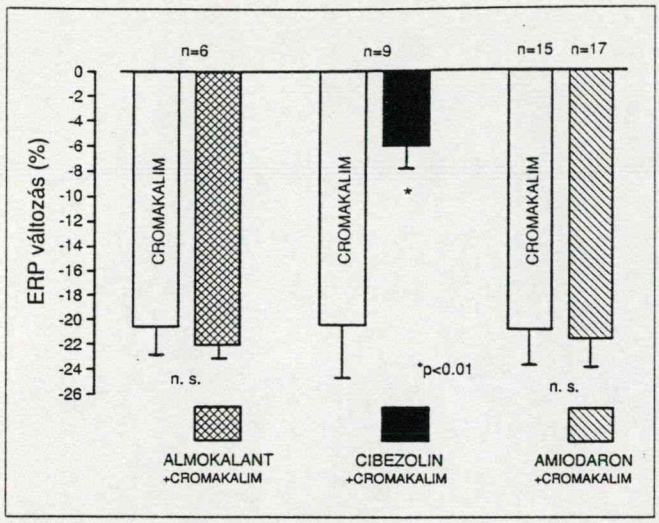
Cromakalim (2 μM/l) alkalmazása után az ERP mintegy 20%-os rövidülését figyeltük meg. Ezt a hatást a cibenzolin jelentős mértékben mérsékelte.



3. ábra. *A cibenzolin (10 μM) frekvenciafüggő hatása az ingerületvezetési időre kamrai munkaizomrostokon. Az abszcisszán az ingerlési frekvenciát az ordinátán az ingerületvezetési időt tüntettük fel. Az ábrán a ± standard error és a szignifikancia szerepel.*



4. ábra. *Az amiodaron kezelés (25 mg/kg/nap, 4 hét + 5 μM a szervfürdőben) frekvenciafüggő hatása az ingerületvezetési időre kamrai munkaizomrostokon. Az abszcisszán az ingerlési frekvenciát, az ordinátán az ingerületvezetési időt tüntettük fel. Az ábrán a ± standard error és a szignifikancia szerepel.*



5. ábra. *Az almokalant (100 nM), cibenzolin (10 μM) és az amiodaron (25 mg/kg/nap, 4 hét + 5 μM a szevfürdőben) hatása a cromakalim (2 μM) által kiváltott ERP rövidülésre. Az ordinátán a kontrollhoz viszonyított %-os ERP változást tüntettük fel. Az ábrán a ± standard error és a szignifikancia szerepel.*

Míg a cromakalim a kontroll mérések során 19,7 ±3,5%-kal (n = 10, p < 0,05) rövidítette az ERP-t, cibenzolin jelenlétében ez a hatás már csak 5,4±1,3 %-os volt, amely különbség statisztikailag is szignifikánsnak bizonyult (p < 0,01, n = 10). Ez az ischaemiában fontos szerepet játszó ATP-függő K-csatornák gátlására utal.

Az almokalanttal és amiodaronnal végzett kísérleteinkben ilyen jellegű hatást nem észleltünk.

MEGBESZÉLÉS

Jelen tanulmány keretében három antiaritmiás szer, az almokalant, a cibenzolin és az amiodaron frekvenciafüggő elektrofiziológiai hatásait vizsgáltuk kutyaszívből izolált jobb kamrai preparátumokon. Az effektív refrakter periódust mindhárom szer szignifikánsan megnyújtotta 1 Hz ingerlési frekvenciánál, mely hatás vélhetően kedvező terápiás értékű a kamrai reentry típusú tachycardiák kezelésében.

Az ingerületvezetést illetően a három szer hatása eltérőnek bizonyult. Az almokalant az ingerületvezetési időt egyetlen alkalmazott ingerlési frekvenciánál sem befolyásolta, amely megegyezik mások korábbi vizsgálataival és arra utal, hogy e szernek nincs kimutatható hatása a nátriumcsatornára (1, 9).

Az amiodaron és a cibenzolin frekvenciafüggő módon nyújtotta az ingerületvezetési időt, amely „use-dependens" Na-csatorna gátlásra utal. A 3. és 4. ábra alapján megállapítható, hogy a két szer ingerületvezetést gátló hatása eltérő jellegű volt. Az amiodaron csak magas ingerlési frekvenciánál gátolta jelentősen az ingerületvezetést, tehát ez a hatás várhatóan csak a fiziológiásnál magasabb szívfrekvenciáknál, ill. korai extraszisztolék fellépésekor lesz igazán számottevő (15, 16). Részben ez a magyarázata annak, hogy az amiodaron Na-csatornagátló akut hatása sokáig ismeretlen maradt. Az amiodaron ezen I/B típusú Na-csatornagátló tulajdonsága feltehetően hozzájárul a szer jól dokumentált antiaritmiás hatásához (14).

A cibenzolin, ellentétben az amiodaronnal, már alacsony és fiziológiás szívfrekvenciánál is gátolta az ingerületvezetést. Ez a hatás terápiás szempontból kevésbé tűnik kedvezőnek, ugyanis a fiziológiás frekvenciánál tapasztalt ingerületvezetési gátlás fokozhatja a proaritmiás komplikációk felléptét.

Kísérleteink alapján megállapítható, hogy a cibenzolin kivédi a cromakalim okozta ERP rövidülést. Ez az ischaemiában fontos szerepet játszó ATP-függő K-csatornák gátlására utal. Akut miokardiális ischaemia során a szívizomsejtek ATP tartalma csökken, ADP tartalma pedig jelentősen növekszik. Ennek hatására az ún. ATP-függő K-csatornák megnyílnak, ami az intracelluláris K kiáramlásához és az extracelluláris tér K-koncentrációjának növekedéséhez vezet. Az ATP-függő K-csatornák megnyílása és az extracelluláris K-koncentráció növekedése egyrészt nagymértékben megrövidíti a szívizomsejtek repolarizációját és refrakter szakát az ischaemiás területen, másrészt gátló hatást fejt ki az ingerületvezetés sebességére, és ezen hatások eredményeként reentry-n alapuló aritmiák forrása lehet (18).

Jelen kísérletünkben a cibenzolin gátolta a cromakalim által megnyitott ATP-függő K-csatornát, amely eredmény megegyezik mások korábbi vizsgálataival (8) és arra utal, hogy az I/A csoportba tartozó cibenzolin terápiás koncentrációban mérsékelni képes mind az intracelluláris K-vesztést, mind az ischaemia aritmogén, akciós potenciált rövidítő hatását (18). Hasonló hatásokról számoltak be mások korábban disopyramiddal, kinidinnel, és prokainamiddal végzett kísérleteik alapján (18).

Ismert, hogy az I/A csoportba tartozó gyógyszerek gátolják a „delayed rectifier" K-áramot (IK) is (14, 18). Így felvetődhet a gondolat, miszerint a cibenzolin a cromakalim okozta ERP rövidülés kivédését részben a „delayed rectifier" K-áram gátlásán keresztül okozta. Ez a lehetőség azonban valószínűtlen, mivel a szelektíven IK-gátlónak tekinthető almokalant nem befolyásolta a cromakalim ERP-t

rövidítő hatását. Az ATP-függő K-csatornák farmakológiai befolyásolásának terápiás szerepe azonban nem tekinthető egyelőre kellőképpen tisztázottnak. Az ATP-függő K-csatornák gátlása egyrészt előnyös lehet a reentry mechanizmussal létrejövő aritmiák kivédésében. Másrészt azonban számos megfigyelés azt látszik igazolni, hogy az ATP-függő K-csatornák megnyílása valószínűleg védekező mechanizmust is jelent az ischaemiás szívizom számára (3, 18). Ez a protektív hatás részben azáltal érvényesül, hogy az ischaemiás területen a csatornák megnyílása okozta repolarizáció és refrakter szak megrövidülés következtében csökken az akciós potenciál platójának időtartama és az alatta a sejtbe áramló Ca ionok mennyisége is, ami a kontraktilitás csökkenéséhez és ezáltal az ischaemiás szív energia igényének mérséklődéséhez is vezet. A másik feltételezett protektív hatás pedig azáltal jön létre, hogy az ATP-függő K-csatornák aktiválódása elősegíti a sejt ATP-készletének megőrzését, ami jelentősen védi a myocardiumot az ischaemiát követő reperfúziós károsodástól (4, 11). Mindezek alapján annak megítélése, hogy a cibenzolinnak, a cromakalim okozta ERP rövidüléssel szembeni gátló hatása kedvező antiaritmiás hatás, vagy inkább hajlamosít további károsodásokra, egyelőre még nem tisztázott kellőképpen.

Összefoglalásként tehát elmondhatjuk, hogy eredményeink alapján jelentős eltérések lehetnek az egyes repolarizációt megnyújtó antiaritmiás gyógyszerek hatásmechanizmusaiban, amelynek hátterében feltehetőleg a szerek különböző K-csatorna gátló hatása áll. Ennek a kezelendő aritmia típusától függően terápiás jelentősége is lehet.

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General Pharmacology
The Vascular System

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Re: MS #909-1-060-99R
Title: "Analysis of the electrophysiological effects of ambasilide, a new antiarrhythmic agent, in canine isolated ventricular muscle and purkinje fibres."

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Editor-in-Chief

ELSEVIER

Analysis of the Electrophysiological Effects of Ambasilide, a New Antiarrhythmic Agent, in Canine Isolated Ventricular Muscle and Purkinje Fibres

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Running title: Electrophysiological effects of ambasilide

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ABSTRACT

The aim of the study was to determine the in vitro rate-dependent cellular electrophysiological effects of ambasilide (10 and 20 μM/L), a new investigational antiarrhythmic agent, in canine isolated ventricular muscle and Purkinje fibres applying standard microelectrode technique. At the cycle length (CL) of 1000 ms, ambasilide significantly prolonged the action potential duration measured at 90% repolarization (APD_{90}) in both ventricular muscle and Purkinje fibres. 10 μM/L ambasilide produced a more marked prolongation of APD_{90} at lower stimulation frequencies in Purkinje fibres (at CL of 2000 ms = 56.0 ± 16.1%; n = 6 versus CL of 400 ms = 15.1 ± 3.7%; n = 6; $p < 0.05$), but in 20 μM/L this effect was considerably diminished (15.2 ± 3.6%; n = 6 versus 7.3 ± 5.1%, n = 6, $p < 0.05$). In ventricular muscle, however, both concentrations of the drug induced an almost frequency-independent lengthening of APD_{90} in response to a slowing of the stimulation rate (in 20 μM/L at CL of 5000 ms = 19.0 ± 1.5%; n = 9 versus CL of 400 ms = 16.9 ± 1.4%; n = 9). Ambasilide induced a marked rate-dependent depression of the maximal rate of rise of the action potential upstroke (V_{max}) (in 20 μM/L at CL of 300 ms = -45.1 ± 3.9%; n = 6; versus CL of 5000 ms = -8.5 ± 3.9%; n = 6; $p < 0.05$ in ventricular muscle) and the corresponding recovery of V_{max} time constant was τ = 1082.5 ± 205.1 ms (n = 6).

These data suggest that ambasilide, in addition to its Class III antiarrhythmic action, which is presumably due to its inhibitory effect on the delayed rectifier potassium current, possesses also I/B type antiarrhythmic properties as a result of the inhibition of the fast sodium channels at high frequency rate with relatively fast kinetics. This latter effect may play an important role in its known less pronounced proarrhythmic ("torsadogenic") potential.

INTRODUCTION

Antiarrhythmic strategies have changed dramatically during the last decade. The disappointing results of the Cardiac Arrhythmia Suppression Trial (CAST) demonstrated that groups of patient treated with flecainide or encainide or moricizine (all are Class I/C agents), and the placebo group exhibited significant differences in survival in patients with ischemic heart disease or postinfarction arrhythmias, with the placebo group faring best (The Cardiac Arrhythmia Suppression Trial (CAST) Investigators, 1989). As a consequence of the CAST, interest has been significantly lost in Class I agents and along with this trend, the therapeutic role of drugs that have antiarrhythmic actions mainly through a prolongation of action potential duration (APD) and refractoriness (Class III agents) have attracted renewed interest (Colatsky et al., 1990; Lynch et al., 1992; Singh, 1993). It was shown in large-scale clinical studies that sotalol (a relatively old Class III drug) is superior to Class I agents in preventing and terminating life-threatening ventricular tachyarrhythmias (Mason & ESWEM Investigators, 1993). The advantage of sotalol has been attributed mainly to its action to prolong APD through the entire heart and this hypothesis has led to research to develop a new series of pure compounds devoid of other associated properties. *d*-Sotalol is one of these new and pure Class III agents. Despite its strong antiarrhythmic effect, however, the SWORD study (Survival with Oral *d*-Sotalol) revealed an increased mortality in post-infarct patients treated with *d*-sotalol (Waldo et al., 1996). 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 (Singh, 1995). Emphasis is therefore shifting to the compounds with a multifaced ("hybrid") pharmacological profile (Link et al., 1996; Podrid, 1995) (with

multiple molecular targets in the framework of the Sicilian Gambit (Task Force of the Working Group on Arrhythmias of the European Society of Cardiology, 1991). 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 (Nattel & Talajic, 1988). Accordingly, development of such drugs is expected to offer favourable new options for antiarrhythmic drug therapy.

Ambasilide (LU 47710, Knoll AG, Ludwigshafen, Germany) 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 (Takanaka et al., 1992; Koidl et al., 1996; Feng et al., 1997). In this study we wished to delineate the frequency-dependent cellular electrophysiological profile of ambasilide in isolated preparations of canine cardiac ventricular muscle and Purkinje fibres by standard microelectrode technique.

METHODS

Papillary muscles were obtained from the right ventricle and free running false tendons of Purkinje fibres were isolated from both ventricles of hearts removed through a right lateral thoracotomy from anaesthetized (sodium pentobarbital 30 mg/kg iv.) mongrel dogs of either sex (8-20 kg). 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). The composition of the Tyrode's solution was (in mM): NaCl 123; KCl 4.7; $NaHCO_3$ 20; $CaCl_2$ 2; $MgCl_2$ 1; and

D-glucose 10. Preparations were oxygenated also in the tissue bath directly. The experiments were carried out by applying 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 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 1000 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), 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}).

The following types of stimulation were applied in the course of the experiments: stimulation with a constant cycle length of 1000 ms; stimulation with different constant cycle lengths (CL) ranging from 300 to 5000 ms (or to 2000 ms in the case of Purkinje fibres in order to avoid spontaneous diastolic depolarization at CLs longer than 2000 ms). To determine the recovery kinetics of V_{max}, extra test action potentials were elicited using single test pulses (S_2) in a preparation driven at a BCL of 500 ms. 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 action potential and were increased progressively.

Ambasilide (LU 47710, Knoll AG, Ludwigshafen, Germany) was diluted in 100% alcohol to make a stock solution of 10 mM/L, and added to the tissue bath in the final concentrations of 10 and 20 μM/L. All measurements were begun 15-30 min after the APD and V_{max} had reached stable values. Stimulus strength was readjusted to twice the diastolic threshold voltage before each drug test. The impalement was maintained in the same cell throughout the procedure including drug application and, whenever possible, during the washout.

All data are expressed as mean ± SEM. Statistical analysis was performed using Student's t test for paired data. The results were considered significant when p was <0.05.

RESULTS

Effects of ambasilide on the action potential parameters in canine ventricular muscle and Purkinje fibres at a BCL of 1000 ms

The effects of 10 and 20 μM/L ambasilide on the action potential parameters of canine ventricular muscle and Purkinje fibres at a BCL of 1000 ms are summarized in Table 1. Ambasilide (10 μM/L) lengthened APD_{50} and APD_{90} significantly in papillary muscles without causing considerable change in the resting membrane potential, action potential amplitude and V_{max}. In Purkinje fibres the same concentration of the drug 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 fibres, 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 fibres, 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 fibre action potential configurations are shown in Figure 1/A and B.

Frequency-dependent effects of ambasilide on action potentials in ventricular muscle and Purkinje fibres

To study the rate-dependent effect of ambasilide on APD_{90}, we stimulated the preparations at CLs ranging from 300 to 5000 ms (or to 2000 ms in the case of Purkinje fibres in order to avoid spontaneous diastolic depolarization at longer CLs). Figure 2 shows the effects of 10 and 20 μM/L ambasilide on APD_{90} as a function of the stimulation cycle lengths in ventricular papillary muscles, while Figure 3 displays those on the same parameter in Purkinje fibres. Although the absolute increase was more pronounced at slow rates (about 100 ms), normalizing the difference between the slow and fast rates as a percentage change 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. 2/A). In contrast, the same concentration of ambasilide exerted a more pronounced increase in APD_{90} in Purkinje fibre preparations at long CLs; the difference tended to disappear at higher stimulation frequencies (CL = 400 ms) (Fig. 3/A). Thus, the APD-rate relations recorded in Purkinje fibres after the application of the drug were considerably steeper when compared with the APD-rate relations obtained from papillary muscle preparations: an increase of the CL from 400 to 2000 ms caused an

additional prolongation of 40.9% in the APD_{90} of Purkinje fibres, but the similar change in CL caused an increase of only 8.7% in papillary muscles.

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. 2/B). In Purkinje fibres, however, the higher concentration of the drug caused less change in APD_{90} than that observed with 10 μM/L. (Fig. 3/B).

By applying stimulation with different constant cycle lengths ranging from 300 to 5000 ms, we also studied the effect of ambasilide on the rate-dependent depression of the maximal rate of rise of depolarization (V_{max}). Figure 4/A shows the relationship between the CL and the inhibition of V_{max} caused by 20 μM/L ambasilide in papillary muscle preparations. The V_{max} depression caused by the drug was strongly cycle length dependent, but significant effect was observed only within the CL range of 300-1000 ms, while at longer CLs V_{max} change was not statistically significant. Examining the same effect in Purkinje fibres, ambasilide exerted negligible depression of V_{max}. (at CL of 400 ms = -3.0 ± 2.7%; n = 6 and at CL of 2000 ms = -2.2 ± 2.0%; n = 6).

At fast stimulation frequencies the drug affected not only V_{max}, but it produced a significant increase also in 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.4/B).

Recovery and onset kinetics of V_{max}

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 5/A shows the recovery of V_{max} in 6 papillary muscle cells in the abscence and presence of 20 μM/L ambasilide. The V_{max} of premature beats elicited once after every 10th basic beat (at a CL of 500 ms) are plotted as a function of the diastolic interval (interval between the 90% repolarization of the basic beat and the upstroke of the premature beat). The restitution curves illustrated in Fig. 5/A show that the drug slowed the recovery of V_{max} with a recovery time constant of τ = 1082.5 ± 205.1 ms (n = 6), which appears to be close to that reported with Class I/B agents.

Thè onset kinetics of V_{max} block induced by 20 μM/L ambasilide was also studied in dog right ventricular papillary muscle. (Figure 5/B). Preparations were continously stimulated at CL of 1000 ms. The stimulation was interrupted for 1 minute and then a train of 40 beats stimuli was applied with cycle length of 400 ms (Figure 5/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 I/B and 1/C antiarrhythmic drugs and was similar to that reported of disopyramide and quinidine (Campbell, 1983b).

DISCUSSION

In the present study we have investigated the actions of 10 and 20 μM/L ambasilide, a novel antiarrhythmic agent, in canine ventricular papillary muscle and Purkinje fibre

preparations. 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 fibres are stimulated at CLs longer than 700 ms. However, the drug exerted a differential effect on APD_{50} in the two types of preparations: in Purkinje fibres 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 fibres at long CLs might contribute to the development of early afterdepolarizations (EADs) and triggered activity, and thus sets the stage for a variety of reentrant arrhythmias, including torsade de pointes (Roden & Hoffman, 1985). The effects of ambasilide proved to be different from those of pure Class III agents: the concentration of 20 μM/L ambasilide that induced the prolongation of APD_{90}, produced a shortening of APD_{50} in Purkinje fibres. Thus 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 fibres, which is probably due to the inhibitory effect of the drug on the slowly inactivating (Carmeliet, 1987) or "window" sodium current (Attwell et al.,1979), 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 fibres versus those in ventricular muscle when the stimulation frequency was changed. In ventricular muscle the drug produced a relatively parallel prolongation of APD_{90} in response to stimulation with different constant cycle lengths. In contrast, lengthening of APD_{90} in Purkinje fibres was more expressed at long CLs. 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 (Hondeghem & Snyders, 1990), is particularly evident in M cells and Purkinje fibres due to the preferential response of these cell types to agents that prolong APD (Antzelevitch & Sicouri, 1994). This feature of most Class III drugs can seriously limit

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 fibres at slow rates leads to a marked increase in the transmural dispersion of repolarization, setting the stage for a variety of reentrant arrhythmias (Antzelevitch et al., 1996. and Surawicz 1989). Our data indicate that both concentrations of ambasilide produced a fairly similar prolongation of the canine ventricular muscle APD_{90} at all rates and therefore did not display reverse use-dependence. Although in 10 μM/L the APD prolonging effect of the drug proved to be clearly reverse use-dependent in Purkinje fibres, this effect was moderate after applying the higher concentration of ambasilide. 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, although because of the difficulties in comparing the results of in vivo and in vitro experiments, the potential therapeutic and proarrhythmic effects of the drug should be assessed in the clinical settings. In this context it would also be of interest, however, to evaluate the effect of the drug also on M cells, a population of cells estimated to comprise at least 40% of the total ventricular myocardial mass in the canine and human ventricle. Due to the inhibitory effect of the drug on both I_{Kr} and I_{Na}, it is probable that an increase in the concentration can induce a shortening of the APD of M cells, as does amiodarone on Purkinje fibres (Sicouri et al., 1997), resulting in a decrease of the transmural dispersion of repolarization.

These findings of the present study in ventricular muscle are consistent with the results of former studies in guinea pig (Zhang et al., 1992; Schreieck et al., 1997), dog (Takanaka et al., 1992) and human ventricular muscle (Weyerbrock et al., 1997) 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} (rapid component of the delayed rectifier potassium current) and other repolarizing currents such as I_{Ks} (slow component of the delayed rectifier potassium current) (Zhang et al., 1992), and, in atrial tissue I_{to} (transient outward current) and I_{so} (sustained outward current) (Koidl, 1996; Feng et al., 1997).

Ambasilide also exhibited additional electrophysiological effects of potential pharmacological significance. Although even the higher concentration of the drug failed to produce a significant change in V_{max} at a CL of 1000 ms, 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. The use-dependent effect on V_{max} (i.e. the largest depressant effect occured at the shortest stimulation cycle length applied) suggests depression of the inactivated fast sodium channels, but this effect is only evident in depolarized tissue, and at high stimulation frequencies (during tachycardia) or at early extrasystoles, without significantly affecting conduction of the action potentials at normal heart rate (Class I/B effect) (Varró et al., 1985a). The findings about the use-dependent effect on V_{max} are in accord with the results of Takanaka et al. (1992), 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 $\underline{\tau} =$ 1082.5 ± 205.1 ms, n = 6). This value appears to be close to the time constant of Class I/B agents (Campbell, 1983a) , and definitely faster than that of Class I/A and Class I/C

compounds (Varró et al., 1985a). 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 (Campbell, 1983a,b) and similar to that repoted of dispyramide (Campbell, 1983a). 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, 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.

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 (The CAST Investigators, 1989).

In this context it is noteworthy that ambasilide seems to share striking similarities with amiodarone in several respects. 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 (Takanaka et al., 1992; Follmer et al., 1994), 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 the chronic treatment with amiodarone, insofar as it either abbreviates phase 2 of the Purkinje fibre action potential (Yabek et al., 1986) or produces markedly less prolongation of APD_{50} in Purkinje fibres than in ventricular muscle (Papp et

al., 1996), 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 (Varró et al., 1985b), 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 (Vaughan-Williams, 1981). Despite all the similarities mentioned, some differences are also present between the two drugs. 1. It has recently been shown that chronic amiodarone exerts a more pronounced effect in papillary muscles than in Purkinje fibres (Papp et al., 1996), whereas ambasilide has opposite effects. 2. Amiodarone exhibits Class I, II, III, IV effects, whereas ambasilide has only Class I/B and III effects. 3. In contrast to ambasilide, the effects of amiodarone are not dose dependent. 4. Chronic amiodarone has been shown to decrease transmural dispersion of repolarization; the effects of ambasilide in this respect remain to be evaluated. 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.

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FIGURE LEGENDS

Figure 1. Action potential recordings from canine ventricular papillary muscles (panel A) and Purkinje fibre strands (panel B) before (control) and after superfusion for 40 minutes with 10 (top) and 20 μM/L ambasilide (bottom).

Figure 2. Rate-dependent effect of 10 (Panel A) and 20 μM/L (Panel B) ambasilide on action potential duration measured at 90% repolarization (APD_{90}) in canine right ventricular papillary muscles. Mean ± SEM values are shown. Asterisk denotes $P < 0.05$ versus control.

Figure 3. Rate-dependent effect of 10 (Panel A) and 20 μM/L (Panel B) ambasilide on action potential duration measured at 90% repolarization (APD_{90}) in Purkinje fibres isolated from both ventricles of the canine heart. Mean ± SEM values are shown. Asterisk denotes $P < 0.05$ versus control.

Figure 4. Rate-dependent inhibition of the maximal rate of depolarization (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 5. 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 (interval between the 90% repolarization of the basic beat and the upstroke of the premature beat) in seconds. Values are shown as mean. For the sake of clarity SEM is not indicated. (Range: 11.4 to 19.4 V/s.)

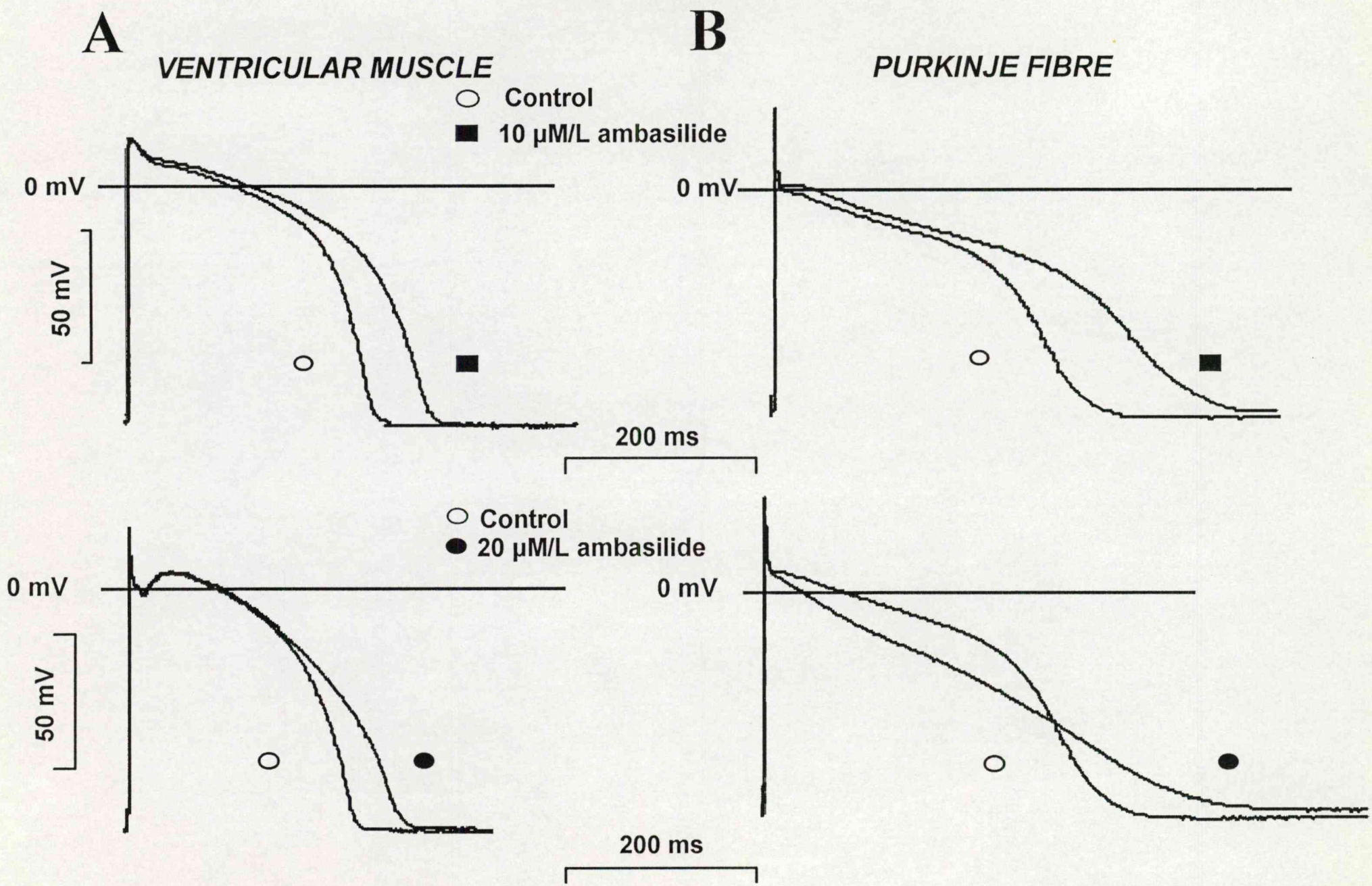
In Panel B following a 1 minute stimulation free period, the V_{max} values of a train of 40 beats at a cycle length of 400 ms are presented. The ordinate indicates the V_{max} values, the abscissa shows the number of the beats in the train. The range of SEM of V_{max} is 3.2 to 12.3 V/s.

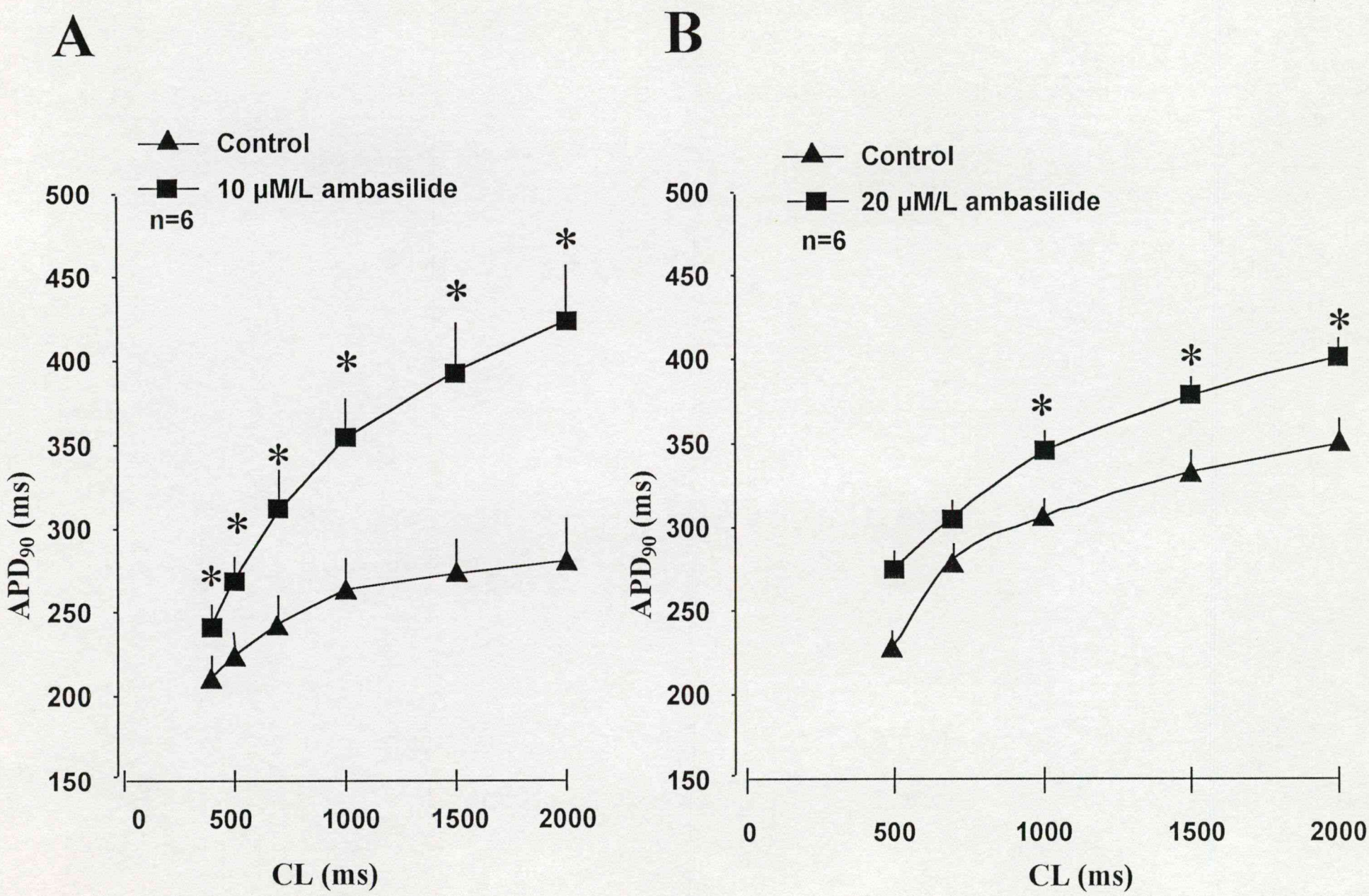
Table 1. Effects of ambasilide on canine action potential parameters at cycle length of 1000 ms

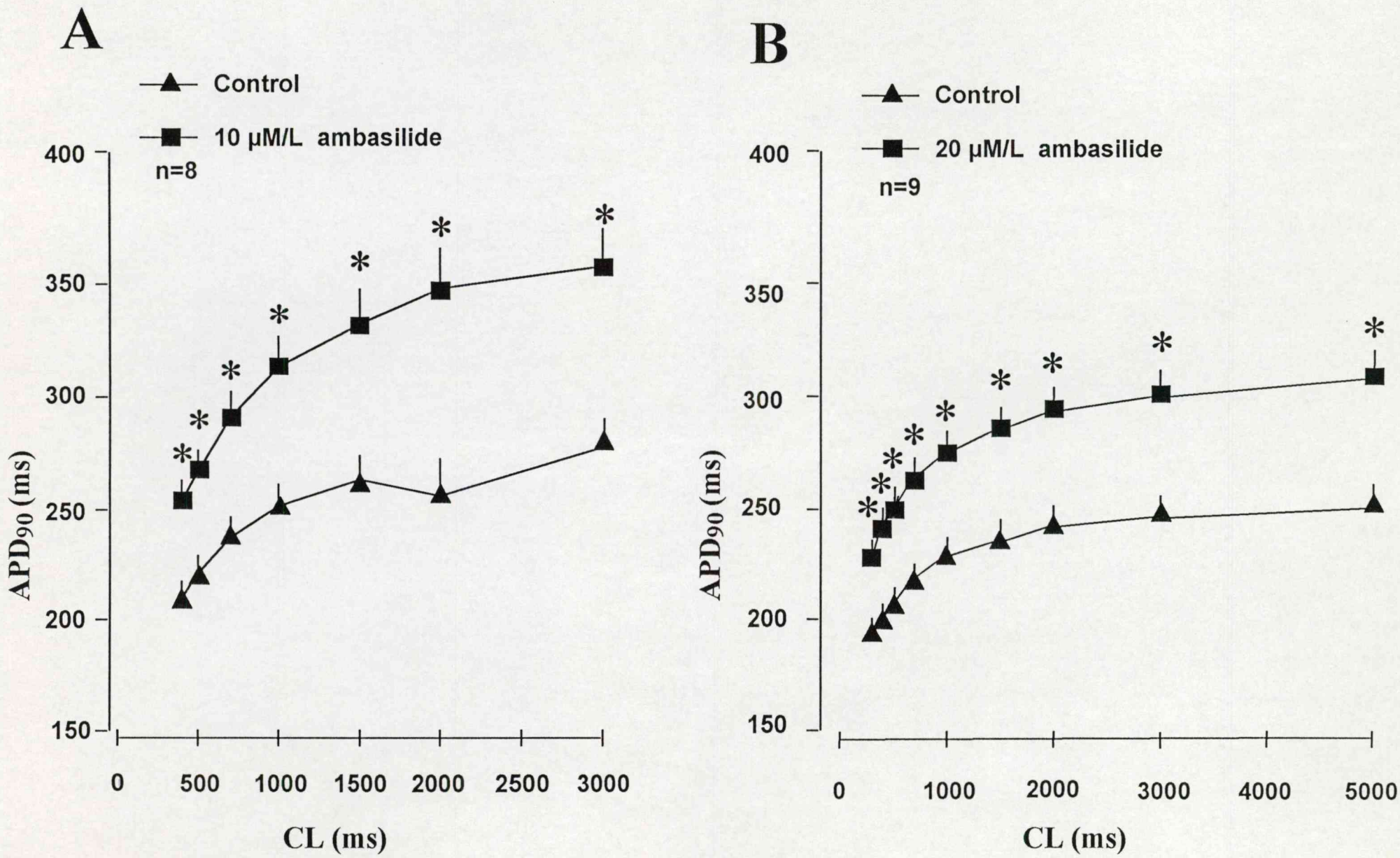
Ventricular muscle	10 μM/L Ambasilide (n=8)		20 μM/L Ambasilide (n=10)	
	Control	Drug	Control	Drug
RP	-88 ±0.6	-90 ±1.1	-85 ± 0.9	-85 ± 0.6
APA	111 ±1.6	112 ±1.6	106 ± 1.1	105 ± 1.1
APD_{50}	219 ±10.4	254 ±13.3*	204 ± 5.6	228 ± 5.9*
APD_{90}	261 ±8.1	323 ± 12.9†	242 ± 5.7	289 ± 6.6†
V_{max}	219 ±29.2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

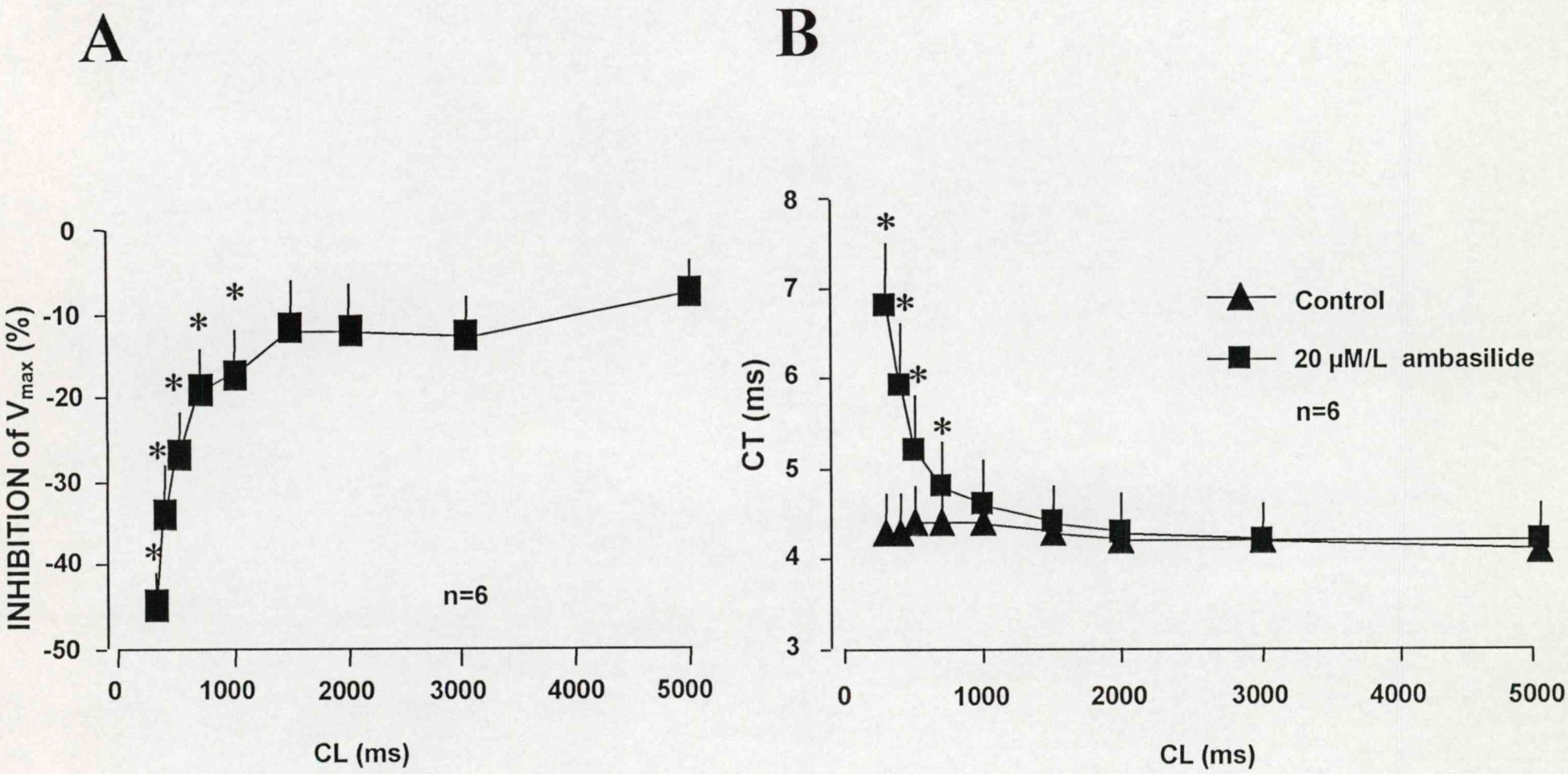
Purkinje fibre	10 μM/L Ambasilide (n=6)		20 μM/L Ambasilide (n=6)	
	Control	Drug	Control	Drug
RP	-89±1.0	-89±1.9	-87 ± 0.6	-85 ± 0.8
APA	112 ±3.5	112 ±2.0	117 ± 1.2	113 ± 2.1
APD_{50}	193 ±16.5	208 ±20.4	236 ± 10.6	145 ± 4.6†
APD_{90}	275 ±16.6	369 ± 19.5†	321 ± 9.9	361 ± 7.2*
V_{max}	416 ±14.0	419 ±22.3	445 ± 14.6	413 ± 15.8

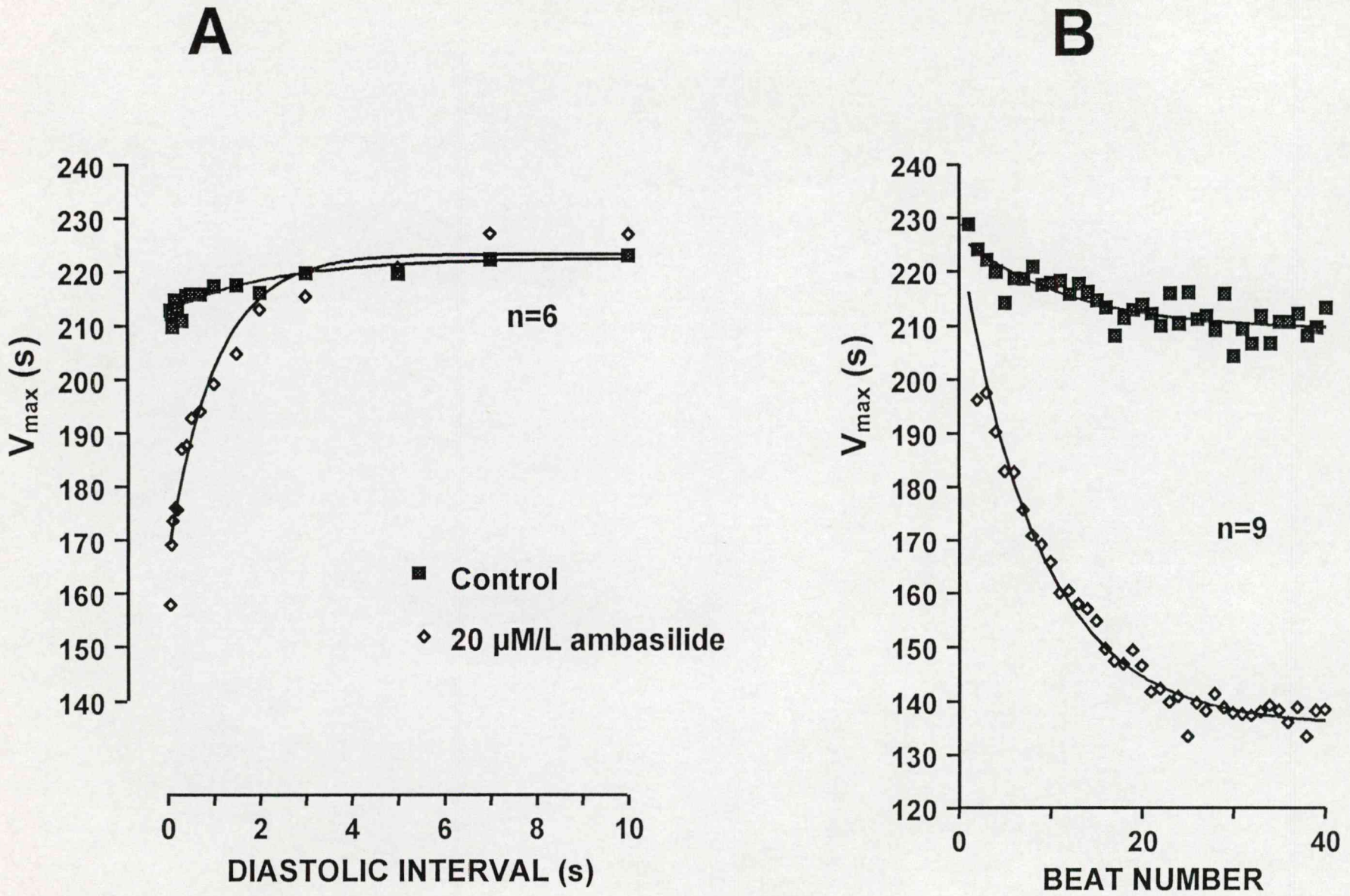
Values are mean ± SEM; RP = resting potential; APA = amplitude of the action potential; APD_{50} , APD_{90} = action potential duration measured at 50 and 90% of repolarization; V_{max} = maximal rate of rise of the action potential upstroke. * $p < 0.05$; † $p < 0.001$.


A
VENTRICULAR MUSCLE
B
PURKINJE FIBRE
Control
10 µM/L ambasilide
0 mV
50 mV
200 ms
Control
20 µM/L ambasilide
0 mV
50 mV
200 ms


A
Control
10 μM/L ambasilide
n=6
500
450
400
350
300
250
200
150
APD90 (ms)
0
500
1000
1500
2000
CL (ms)
B
Control
20 μM/L ambasilide
n=6
500
450
400
350
300
250
200
150
APD90 (ms)
0
500
1000
1500
2000
CL (ms)


A
Control
10 μM/L ambasilide
n=8
400
350
300
250
200
150
APD90 (ms)
0
500
1000
1500
2000
2500
3000
CL (ms)
B
Control
20 μM/L ambasilide
n=9
400
350
300
250
200
150
APD90 (ms)
0
1000
2000
3000
4000
5000
CL (ms)


A
B
INHIBITION of V_{max} (%)
0
-10
-20
-30
-40
-50
0
1000
2000
3000
4000
5000
CL (ms)
n=6
CT (ms)
8
7
6
5
4
3
Control
20 μM/L ambasilide
n=6
CL (ms)


A
B
240
230
220
210
200
190
180
170
160
150
140
V_{max} (s)
n=6
Control
20 μM/L ambasilide
0
2
4
6
8
10
DIASTOLIC INTERVAL (s)
n=9
130
120
20
40
BEAT NUMBER

III

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Comparison of the cellular electrophysiological characteristics of canine left ventricular epicardium, M cells, endocardium and Purkinje fibres

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ABSTRACT

Electrophysiological differences among M cells, epicardium, endocardium and Purkinje fibres of the canine ventricle were studied over a wide range of stimulation cycle lengths, and the pharmacological response of these cell types to the sodium channel blocker tetrodotoxin, calcium channel blocker nifedipine and ATP-sensitive potassium channel activator pinacidil was compared. The experiments were carried out by applying standard intracellular microelectrode technique in isolated dog left ventricular preparations. The results confirmed the existence of M cells in the canine ventricle, in addition, the distribution of the rate of rise of the action potential upstroke and action potential amplitude values reflecting probably the inhomogeneity of the fast sodium current in these cells was revealed. It was also demonstrated that M cells differ from Purkinje fibres in some aspects which were not expected from previous investigations: (1) The early portion of the action potential duration restitution curve in M cells is more similar to that of endocardial and epicardial cells than to Purkinje fibres. (2) The plateau phase of the action potentials in Purkinje fibres developed at a more negative potential range than that in the other cell types studied. (3) The pharmacological response to tetrodotoxin and pinacidil in M cells resembles to that in the endocardial and epicardial cells more than in the Purkinje fibres. Our results provide further evidence in support of the existence of M cells but also indicate that there are important electrophysiological as well as pharmacological differences between M cells and Purkinje fibres.

Keywords action potential duration, dog ventricular preparations, electrophysiology, rate dependence, rate of rise of the action potential upstroke.

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Until recently, most investigations in the cardiac electrophysiology and pharmacology of the ventricles focused on two main cell types, namely those of the ventricular endocardium and Purkinje fibres. Most of our electrophysiological and pharmacological knowledge about ventricular myocardium stemmed from studies using endocardial tissues. Data obtained from endocardium were often generalized and considered to be representative of ventricular myocardium as a whole.

Recent studies, however, have described important regional differences in the electrophysiology and pharmacology of ventricular myocardium in mammalian hearts (Antzelevitch *et al.* 1991, Antzelevitch & Sicouri 1994) and provided data supporting the existence of at least four functionally distinct cell types in the ventricles including epicardial, midmyocardial (M), endocardial and Purkinje cells. Several previous investigations have highlighted the electrophysiological distinctions between ventricular endocardium and epicardium, demonstrating different, and sometimes opposite responses of these two cell types to pharmacological agents and pathophysiological states in a number of species (Gilmour & Zipes 1980, Litovsky & Antzelevitch 1988, 1990, Fedida & Giles 1991, Krishnan & Antzelevitch 1991, Tande *et al.* 1991, Furukawa *et al.* 1991, 1992, Wettwer *et al.* 1994). Midmyocardial or M cells, a unique and distinct subpopulation of cells in the deep subepicardial layers of the canine ventricle have been identified by Sicouri and Antzelevitch, and were found to have electrophysiological features intermediate between those of myocardial and Purkinje cells (Sicouri & Antzelevitch 1991a) and pharmacological

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responsiveness different from that of either epicardium or endocardium (Sicouri & Antzelevitch 1991b, 1993, Sicouri *et al.* 1997).

As the electrophysiological and pharmacological properties of M cells have not been fully elucidated, the present work was designed 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 to compare the pharmacological response of these cell types to the sodium channel blocker tetrodotoxin (TTX 2 μM L^{-1}), calcium channel blocker nifedipine (2 μM L^{-1}) and ATP-sensitive potassium channel activator pinacidil (10 μM L^{-1}). As the majority of studies so far available on this topic suggest that M cells bear outstanding resemblance with Purkinje fibres and share only minor similarities with ventricular muscle fibres in the epicardium and endocardium, we have focused our investigations especially on the electrophysiological and pharmacological differences between midmyocardial and Purkinje cells of the canine ventricle.

METHODS

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^{-1} i.v.) mongrel dogs of either sex (8–12 kg). 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 (1991a). Briefly, the left ventricle was cut from the base to the apex with scissors, and small cubes of transmural slices (= 1.5 × 1.0 × 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 free wall were then carefully cut by razor blade shavings, to obtain final preparations which were 10–15 mm long, 10 mm wide and 1 mm thick. M cells were recorded from slices located 2–5.2 mm from the epicardial surface. As 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 fibres 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 h while being superfused with oxygenated (95% O_2:5% CO_2) Tyrode's solution (flow = 4–5 mL min^{-1}) warmed to 37 °C (pH 7.3 ± 0.5) and containing (in mmol L^{-1}) 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.

Action Potential Recordings

The experiments were carried out by applying 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 at one or more sites with the use of glass capillary microelectrodes filled with 3 M KCl (tip diameter <1 μM, resistance 10–25 MΩ). The microelectrodes were coupled through a 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 1000 V s^{-1}. 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), 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 rest of the paper, APD refers to APD_{90}. In the case of papillary muscles, recordings were always made from the apical region, known to be devoid of Purkinje fibres. 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 5000 ms in the case of Purkinje fibres 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 ms. 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.

Drugs

Drugs were diluted in normal Tyrode's solution to obtain the following final concentrations: 10 μM L^{-1} pinacidil (GYKI, Budapest), 2 μM L^{-1} tetrodotoxin (Sigma, St. Louis, USA), 2 μM L^{-1} nifedipine (Sigma, St. Louis, USA). All measurements were begun 15–30 min after the APD and V_{max} reached stable values. Stimulus strength was readjusted to twice the diastolic threshold voltage before each drug test. The impalement was maintained in the same cell throughout the procedure including drug application and, whenever applicable, during the washout.

Statistics

All data are expressed as mean ± SEM. Statistical analysis was performed using the non-parametric form of analysis of variance (ANOVA) coupled with the Mann-Whitney and Bonferroni procedures, and Student's *t*-test for paired or unpaired data, as indicated. The results were considered significant when P was <0.05.

RESULTS

Action potential configurations and characteristics

Action potentials recorded from preparations isolated from the epicardial, midmyocardial, endocardial regions and from Purkinje fibres of the canine left ventricle are illustrated in Fig. 1. 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 fibres 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 fibres with respect to phase 3 repolarization, showing a greater prolongation of the action potential with slowing of the stimulation rate. At the BCL of 300 ms APDs recorded at the four different sites are similar. In all the four cell types, APD increases progressively as the rate is slowed from the BCL of 300 to 1000 and 5000 ms, but M cells and Purkinje fibres display a longer APD and a more accentuated rate dependence than the epicardial and endocardial cells. It is worth mentioning that the plateau phase of the action potentials in Purkinje fibres 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 fibres (17.1 ± 1.1 mV).

Table 1 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^{-1}). It was found that M cells and Purkinje fibres

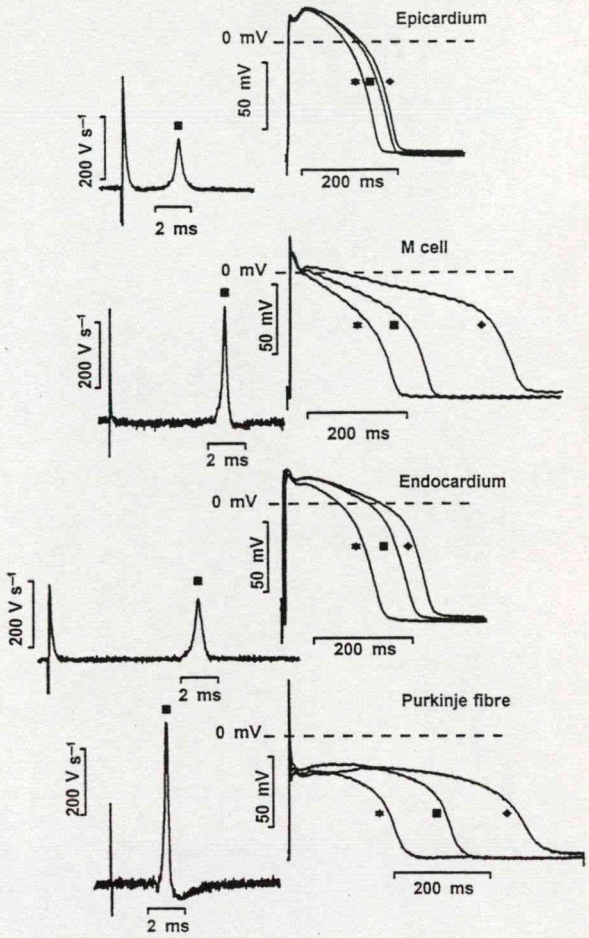

Figure 1 Transmembrane action potentials (right panel) recorded under steady-state conditions at basic cycle lengths of 300 (★), 1000 (■) and 5000 ms (◆) from epicardium, midmyocardium (M cell), endocardium and Purkinje fibres isolated from the canine left ventricle. The maximal rate of rise of the action potential upstroke (V_{max}) (left panel) recorded from the respective regions of the left ventricle at a basic cycle length of 1000 ms (■).

displayed a resting membrane potential more negative than that in epicardial and endocardial cells. Action potential amplitude in Purkinje fibres was considerably higher than that observed in the different types of ventricular muscle fibres. The action potential duration 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 fibres. 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 fibres. Interestingly, the distribution of V_{max} values recorded in M cells demonstrated a marked variance in magnitude. As endocardial and epicardial cells in normal conditions have a V_{max} value smaller

Table 1 Action potential parameters of endocardial, M, epicardial cells and Purkinje fibres recorded from canine left ventricular preparations at basic cycle length of 1000 ms

	Endocardium ($n = 28$)	M cell ($n = 37$)	Epicardium ($n = 29$)	Purkinje fibre ($n = 20$)
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} (Vs^{-1})	176.9 ± 7.0*	284.0 ± 17.5†	154.5 ± 6.6	505.0 ± 32.7*†‡

Values are mean ± SEM; RP = resting potential; APA = amplitude of the action potential; APD_{90} = action potential duration at 90% repolarization; V_{max} = maximal rate of rise of the action potential upstroke. Significance was determined by the non-parametric 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

than 300 V s^{-1}, we arbitrarily divided the V_{max} values of 37 individual M cells into two subgroups, under and above 300 V s^{-1}. In 14 of 37 preparations it was found that the V_{max} value was greater than 300 V s^{-1} with a mean of 397.7 ± 18.5 V s^{-1}, while in 23 preparations V_{max} was measured less than 300 V s^{-1} 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^{-1}, 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 bore similarity with the variance of V_{max}. These features of action potential parameters in M cells are demonstrated in Table 2.

Table 2 Distribution of action potential parameters in M cells recorded from canine left ventricle preparations at basic cycle lengths of 1000 and 10 000 ms

	V_{max} > 300 V s^{-1} ($n = 14$)	V_{max} < 300 V s^{-1} ($n = 23$)
BCL of 1000 ms		
V_{max} (V s^{-1})	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} (ms)	260.8 ± 8.2	257.4 ± 5.2
BCL of 10 000 vs 1000 ms		
APD_{90} prolongation (%)	36.0 ± 6.4	47.7 ± 5.5

Data presented are mean ± SEM; BCL = basic cycle length; V_{max} = maximal rate of rise of the action potential upstroke; RP = resting potential; APA = amplitude of the action potential; APD_{90} = action potential duration at 90% repolaraization. Significance was determined by Student's test for unpaired data. * $P < 0.001$; † $P < 0.05$

Rate dependence of action potential duration under steady-state conditions

The main characteristic of M cells which differentiates them from the other ventricular cell types and makes them more similar to Purkinje fibres, 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 fibres in order to avoid spontaneous diastolic depolarization at cycle length of 10 000 ms). Figure 2 shows the effects of the stimulation cycle lengths on APD_{90} of epicardial, M and endocardial cells as well as of Purkinje fibres. 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. Thus, the APD-rate relations recorded in M cells are considerably steeper when compared with the APD-rate relations obtained from endocardial and epicardial preparations. In the left ventricle, an increase of the BCL from 1000 to 10 000 ms caused a 41% increase in

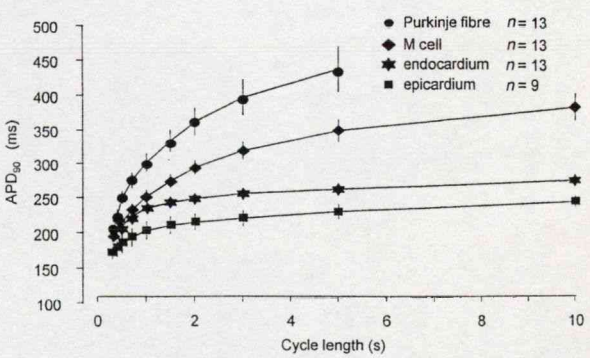

Figure 2 Rate dependence of action potential duration measured at 90% repolarization (APD_{90}) in epicardial cells (■), M cells (◆), endocardial cells (★) and Purkinje fibres (●) of the canine left ventricle. Mean ± SEM values are shown for basic cycle lengths (BCLs) of 300, 500, 700, 1000, 1500, 2000, 3000, 5000 and 10 000 ms.

the APD_{90} of M cells, but an increase of only 14% in epicardial and 13% in endocardial fibres. In Purkinje fibres, a deceleration of rate from 1000 to 5000 ms produced a dramatic prolongation of APD similar to that of M cells (35%). Thus, the action potential duration rate relations are remarkably more prominent in M cells than those in epicardium or endocardium, and similar to those in Purkinje fibres, which are known to prolong more excessively relative to the other cell types when stimulation rate is slowed.

Restitution of action potential duration

The results so far presented indicate important distinctions between M cells and other ventricular muscle cells with respect to rate-dependent changes in APD under steady-state conditions but indicate similarities in the rate-dependence of M cells and Purkinje fibres. As the determinants of APD are known to differ between steady-state and non-steady-state simulation conditions, we examined and compared the characteristics of restitution of this parameter in epicardial, endocardial, M cells and Purkinje fibres.

The four curves illustrated in Fig. 3 depict the restitution of APD in 15 midmyocardial, 13 endocardial, 11 epicardial cells and in 16 Purkinje fibres isolated from the left ventricle. The APD_{90} of premature beats elicited once after every 10th basic beat (at BCL of 1000 ms) are plotted as a function of the diastolic interval (interval between the 90% repolarization of the basic beat and the upstroke of the premature beat). The APD restitution curves illustrated in Fig. 3 show

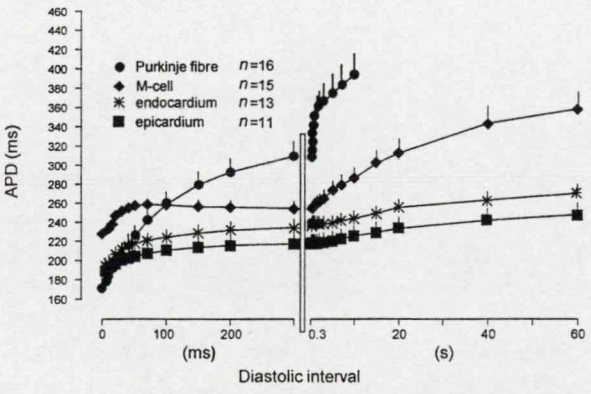

Figure 3 Restitution of action potential duration at 90% repolarization (APD_{90}) in epicardium (■), endocardium (*), M cells (◆) and Purkinje fibres (●) isolated from the canine left ventricle. Basic cycle length was 1000 ms. The ordinate indicates APD_{90} values of the extrasystoles, elicited at progressively increasing diastolic intervals, in ms. The abscissa shows the diastolic intervals (interval between the 90% repolarization of the basic beat and the upstroke of the premature beat). 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.

marked differences among the four cell types. The restitution curves in endocardial and epicardial fibres 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 fibres the fast component of restitution had a τ_{fast} of 17.4 ms (A_{fast} = 29.9 ms) but, as considerably different from epicardial and endocardial fibres, the amplitude of the slow component was relatively big (A_{slow} = 104.1 ms) with similar kinetic parameter (τ_{slow} = 23.8 s). Purkinje fibres, as displayed in Fig. 3, 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.

We also determined and compared 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 in all four types of tissue. As Fig. 4 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 fibres, 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%, 9.5 ± 1.9% and 7.6 ± 2.0%, respectively), while the same parameter in Purkinje fibres showed a marked reduction (46.3 ± 1.6%). Regarding the change in the APD of

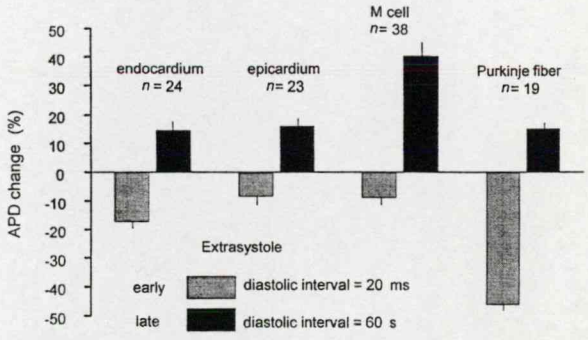

Figure 4 Cycle length dependent action potential duration (APD) at early (20 ms) and late (60 s) diastolic intervals (interval between the 90% repolarization of the basic beat and the upstroke of the premature beat) in endocardium, epicardium, M cells and Purkinje fibres of the canine left ventricle. 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. Mean ± SEM values are presented.

extrasystoles elicited at late diastolic intervals (60 s), the pattern of APD changes differed from that observed in case of extrasystoles with short diastolic intervals. Namely, we found a great prolongation in M cells (40.0 ± 4.6%) and in Purkinje fibres (15.4 ± 1.7%), but only a slight increase in endocardial and epicardial cells (16.7 ± 2.6% and 15.3 ± 3.1%, respectively). As far as the prolongation of APD of extrasystoles recorded in Purkinje fibres 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 fibres, 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.

Pharmacological responses

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 TTX (2 μM L^{-1}), nifedipine (2 μM L^{-1}) and pinacidil (10 μM L^{-1}). Figure 5 illustrates the results of 72 experiments (3 × 24), in which the effects of the three agents on the action potential duration of the preparations were examined at the BCL of 1000 ms.

It was found that the sodium channel inhibitor tetrodotoxin shortened APD in both M cells and Purkinje fibres, but it did not influence this parameter in epicardial and endocardial cells significantly. Figure 5 demonstrates that although action potential duration was abbreviated in both types of tissue, TTX was more effective in shortening APD in Purkinje fibres than in midmyocardial cells (52.8 ± 5.5 and 8.2 ± 4.2%, respectively). These findings suggest a weaker block of the sodium 'window' current or slowly inactivating tetrodotoxin-sensitive sodium current in M cells than in Purkinje fibres.

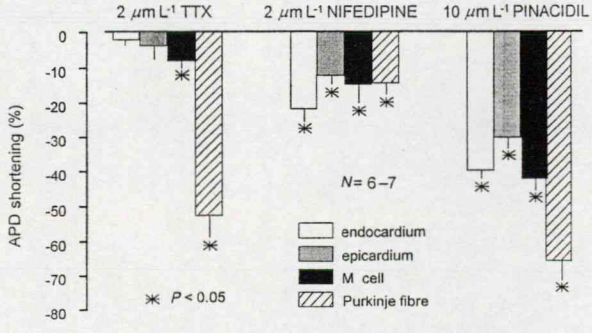

Figure 5 Block diagram illustrating the action potential duration (APD) shortening effects of tetrodotoxin (TTX 2 μM L^{-1}), nifedipine (2 μM L^{-1}) and pinacidil (10 μM L^{-1}) on canine left ventricular endocardium (white), epicardium (grey), M cells (black) and Purkinje fibres (striped columns) at a basic cycle length 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.

The calcium channel blocker nifedipine produced a significant reduction in action potential duration 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 fibres was abbreviated to a larger extent than APD in endocardial, epicardial and M cells.

DISCUSSION

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 (Sicouri & Antzelevitch 1991a). 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 fibres 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 fibres, (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 fibres, (3) the pharmacological response of M cells to TTX or pinacidil resembles the endocardial and epicardial cells more than the Purkinje fibres.

Solberg *et al.*, studying action potentials in different regions of transmural papillary muscle slices obtained from canine ventricle, were the first to describe that cells located in the deeper myocardial layers may differ from surface muscle cells in electrophysiological characteristics (Solberg *et al.* 1974). Some 17 years later 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 (Sicouri & Antzelevitch 1991a,b, 1993, 1995, Liu *et al.* 1993, Liu & Antzelevitch 1995, Anyukhovsky *et al.* 1996, El-Sherif *et al.* 1996, Sicouri *et al.* 1997), guinea-pig (Sicouri *et al.* 1996), rabbit (Weirich *et al.* 1996), and human ventricles (Drouin *et al.* 1995). These cells are distinguished chiefly 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 action potential duration (APD). This feature suggests that the currents underlying repolarization in these cells are different from those of epicardium and endocardium, and similar to those in Purkinje fibres. 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 fibres (Sicouri & Antzelevitch 1991a). 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 fibres (higher V_{max}, steeper APD-rate relation).

Our findings further delineate the distinctions among cells spanning the wall of the canine left ventricle.

Action potential characteristics

Action potentials recorded in the four tissues of the canine ventricle exhibit marked differences in morphology. Prominent among these is the presence of an expressed phase 1 in epicardial, M and Purkinje cells but less in endocardial fibres. In our experiments we found the phase 1 amplitude of epicardial cells less pronounced than observed in earlier studies (Sicouri & Antzelevitch 1991a) which might be due to the smaller size and younger age of the dogs we used. Besides from differences in the early phases of the action potential, a significant distinction exists 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 fibres relative to epicardial and endocardial cells with the slowing of rate. Also, Purkinje fibres 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 (1991a), 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. 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 fibres in response to a slowing of the stimulation rate. Examining the extent of prolongation when BCL was increased from 1000 to 10 000 ms, we established that cells of both groups displayed a marked prolongation of APD typical to M cells. This observation suggests that the cells studied have characteristics ascribed to M cells. 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.

Rate dependence of action potential duration under steady-state conditions

The rate dependence of the action potential duration 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 fibres. Steady-state rate-dependence of APD in the four types of tissue qualitatively resembled one another, i.e. at progressively longer basic cycle lengths the APD of all the four cell types increased. However, in concordance with the report of Sicouri & Antzelevitch (1991a), 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 fibres, 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 (Antzelevitch *et al.* 1989) as stimulation rate is slowed. This heterogeneity provides an important substrate for a variety of re-entrant arrhythmias, including intramural re-entry and torsade de pointes (TdP), and regarding that M cells are estimated to constitute at least 40% of the total ventricular myocardial mass (Sicouri *et al.* 1994), their possible role in arrhythmogenesis is especially of great significance.

Based on the available data on dispersion of repolarization within the ventricle, Antzelevitch suggested that as M cells may be the most abundant cell population in the ventricles and may represent the true working myocardial cells of these cardiac chambers (Antzelevitch 1997), 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 (Antzelevitch 1997). 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 (Yan & Antzelevitch 1996, Chauhan *et al.* 1996). In patients treated with drugs exhibiting Class III anti-arrhythmic 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 (Antzelevitch *et al.* 1996). The implication of this might be an arrhythmic substrate capable of maintaining both monomorphic and polymorphic arrhythmias.

Restitution of action potential duration

The remarkable distinction between the restitution of APD in M cells and Purkinje fibres represents an important electrophysiological difference between the two types of fibre, which has not been described yet. The ionic nature of the restitution of APD is not well understood. The early fast components most likely depend on the deactivation and reactivation of different trans-membrane ion currents such as the inward calcium current, the transient outward and the slow and fast components of the delayed outward potassium currents. The slow phase of restitution may relate to the accumulation or depletion of K^+ and Na^+ or Ca^{2+} in the extracellular and intracellular space influencing sarcolemmal ion pumping mechanisms. To explore the differences in the restitution of APD among the four ventricular tissue types needs further research with more direct experimental methods like the patch-clamp technique in order to get deeper insight into these interesting questions.

Pharmacological responses

A significant consequence of the prominent electrophysiological differences among the four functionally distinct cell types in the ventricles is that they show different – in some cases opposite – responses to a wide variety of pharmacological agents (Litovsky & Antzelevitch 1990, Antzelevitch *et al.* 1991, Furukawa *et al.* 1991, Krishnan & Antzelevitch 1991, Sicouri & Antzelevitch 1991b, 1993, Antzelevitch & Di Diego 1992, Sicouri *et al.* 1997). 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 (Attwell *et al.* 1979, Coraboeuf *et al.* 1979, Noma 1983, Noma & Shibasaki 1985, Kimura *et al.* 1987, Gotoh *et al.* 1991), *in vitro* investigations of the same interventions are limited in epicardial (Kimura *et al.* 1987, Antzelevitch *et al.* 1991, Furukawa *et al.* 1991, Gotoh *et al.* 1991, Krishnan & Antzelevitch 1991) and especially in M cells (Eddlestone *et al.* 1996). Therefore, we compared the pharmacological response of the four tissue types to the sodium channel blocker tetrodotoxin (TTX 2 μM L^{-1}), calcium channel blocker nifedipine (2 μM L^{-1}), and ATP-sensitive potassium channel activator pinacidil (10 μM L^{-1}).

Our results show that after 10 min of exposure to 2 μM L^{-1} TTX, APD was shortened in both M cells and Purkinje fibres 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 fibres, it has to be emphasized that the degree of APD abbreviation was more accentuated in Purkinje fibres than in M cells, suggesting a weaker contribution of the sodium current to repolarization in M cells than in Purkinje fibres, findings showing M cells to be more akin to epicardial and endocardial cells.

Studying the effect of the organic calcium channel blocker nifedipine (2 μM L^{-1}), it was found that it reduced APD in all the four tissue types significantly without displaying remarkable difference with respect to the extent of abbreviation in any of the preparations.

We also assessed and compared the pharmacological effects of pinacidil in the four isolated ventricular preparations. Pinacidil is known to augment ATP-regulated potassium current ($I_{K\text{-}ATP}$) in cardiac tissues (Escande *et al.* 1989), the activity of which is normally inhibited by physiological levels of intracellular ATP. Our data show that the relatively high concentration (10 μM L^{-1}) of the potassium channel activator pinacidil caused a significant abbreviation of repolarization in all the four tissue types. Nevertheless, APD was shortened more excessively in Purkinje fibres than in ventricular muscle fibres. Thus we can conclude that, similar 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 fibres.

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IV

Cardiologia Hungarica

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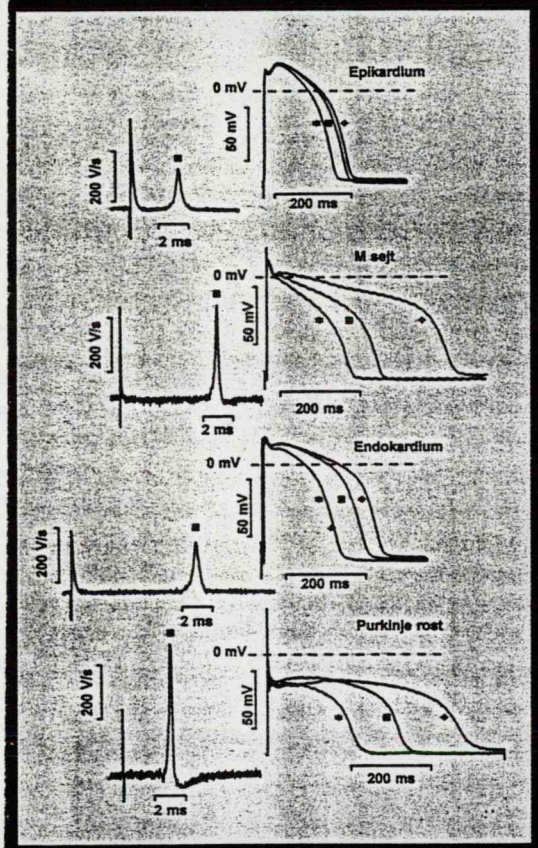

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ÖSSZEFOGLALÓ KÖZLEMÉNY

BALÁTI BEÁTA, VARRÓ ANDRÁS, PAPP GYULA

Az M sejtek elektrofiziológiai jellemzése: élettani, farmakológiai és klinikai jelentőségük

Kulcsszavak: *M sejt • repolarizáció frekvenciafüggése • kamrai diszperzió • aritmogenezis • EKG*

Összefoglalás: Experimentális in vitro és in vivo vizsgálatok igazolták az un. M sejtek jelenlétét számos faj kamrai mély szubepikardiális régiójában. Ezen elektrofiziológiai és farmakológiai sajátságaiban különleges típusú sejteknek döntő szerep tulajdonítható a kamrafalon belüli repolarizációs diszperzió létrejöttében és ezáltal a ritmuszavarok patomechanizmusában, főként az intramurális reentry és triggerelt aktivitás kialakulásában. A szerzők az M sejtek szerepére koncentrálva a legújabb kutatási eredmények és koncepciók alapján adnak áttekintést a szívkamrákat alkotó szövet- és sejttípusok között fennálló elektrofiziológiai és farmakológiai heterogenitás jellemzőiről és feltárják azok megnyilvánulásait az EKG-n, illetve élettani és kóros állapotokban. Irodalmi és saját vizsgálati eredményeik alapján arra következtetnek, hogy a kamrafalon belüli elektromos inhomogenitásra irányuló kutatások a kísérletes és klinikai aritmiák patomechanizmusának és az antiaritmiás gyógyszerek hatásmódjának jobb megértését eredményezhetik.

Keywords: *M cells • rate dependence of repolarization • ventricular dispersion • arrhythmogenesis • ECG*

Summary: Several in vitro and in vivo investigations have provided data supporting the existence of M cells in the deep subepicardial layers of the ventricles in a number of species. Characterized by unique electropharmacological features, this subpopulation of cells is regarded to have a significant role in creating dispersion of repolarization in the ventricular wall and thus contribute importantly to arrhythmogenesis, in particular to intramural reentry and triggered activity. Focusing on M cells, the authors summarize recent findings and concepts concerning the electrophysiological and pharmacological heterogeneity of different cell and tissue types found within the ventricles and explore how these differences may contribute to electrocardiographic manifestations, as well as physiological and pathophysiological function. On the basis of literarary data and of their own results they conclude that studying the electrical inhomogeneity within the ventricular wall may provide a better understanding of the pathophysiological processes that give rise to cardiac rhythm disturbances and the mechanisms by which antiarrhythmic agents act to suppress and in some cases aggravate arrhythmias.

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Munkahely: Szent-Györgyi Albert Orvostudományi Egyetem Farmakológiai és Farmakoterápiai Intézet (intézetvezető: Dr. Papp Gyula akadémikus, egyetemi tanár)

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A közelmúltig az emlős szív kamrai munkaizomrostjainak elektrofarmakológiai jellemzőit viszonylag homogénnek tartották. Ez valószínűleg azzal állt összefüggésben, hogy a kamrai munkaizomrostok celluláris elektrofiziológiai vizsgálatát túlnyomórészt endokardiális preparátumokon (papilláris izom, trabekuláris izom) végezték. Az ezen kísérletekben nyert adatokat gyakran általánosították, és a kamrai szívizomzat egészére jellemzőnek tekintették. Újabb kutatások azonban jelentős regionális különbségeket tártak fel az emlős szív kamrai munkaizomzatának egyes rétegei között elektrofiziológiai és farmakológiai szempontból egyaránt (6, 7), kimutatva három funkcionálisan elkülönülő sejttípus – az epikardiális sejtek, a szívizom középső rétegéből származó M sejtek, és az endokardiális sejtek – jelenlétét a kamrafalban. Több korábbi tanulmány már beszámolt ugyan számos faj endokardiuma és epikardiuma között fennálló celluláris elektrofiziológiai különbségekről, és igazolta ezen két szövettípus eltérő, néha egymással ellentétes válaszát farmakológiai szerekre és kóréletteni állapotokra (26, 28, 29, 30, 36, 39, 41, 63, 71), mégis az M sejtek megismeréséig e közlések nem kaptak kellő hangsúlyt az irodalomban. A szívizom mélyebb rétegeiből származó M sejteket, melyek a kutya kamra mélyebb szubepikardiális rétegének sajátságos sejttípusát képezik, nemrégiben írta le Sicouri és Antzelevitch, és úgy találták, hogy ezek elektrofiziológiai szempont-

ból átmenetet képviselnek a kamrai munkaizomsejtek és Purkinje rostok között (51), míg farmakológiai érzékenységüket tekintve, mind az epikardium, mind az endokardium sejtjeitől eltérően viselkednek (52, 53, 57). Az M sejtek felfedezése és leírása számos elektrofiziológiai, farmakológiai, patofiziológiai és elektrokardiográfiai jelenség újraértékelését, és egyúttal jobb megértését eredményezte.

Jelen munkánkban az M sejtek szerepére koncentrálva, összefoglaljuk azokat a legújabb kísérleti eredményeket, melyek feltehetőleg elősegítik a kamrákban fennálló elektromos heterogenitás pontosabb megértését, továbbá megpróbálunk rávilágítani arra, hogy a kamrai sejttípusok elektrofiziológiai jellemzőiben és farmakológiai válaszkészségében megtalálható eltérések miképpen nyilvánulnak meg az EKG-n (U-hullám), valamint különböző fiziológiai és patofiziológiai funkciókban.

A KAMRAFAL ELEKTROFIZIOLÓGIÁJA

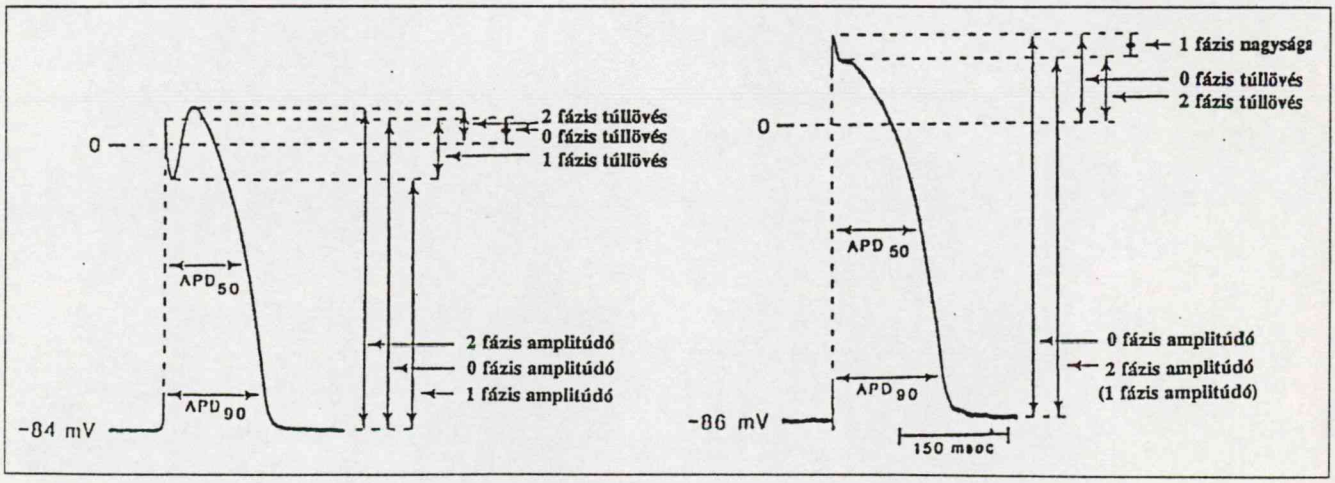
Epikardiális és endokardiális sejtek

Korábban celluláris szintű elektrofiziológiai vizsgálatok céljára főként a szív endokardiális, ritkán az epikardiális felszínéről származó preparátumokat használtak. Ez egyrészt a szív felszíni szöveteinek könnyebb hozzáférhetőségével, másrészt az ép (nem vágott) felszínről történő regisztrálás nyilvánvaló előnyével magyarázható. Ennek eredményeképpen jelentős mennyiségű megfigyelés áll rendelkezésünkre számos faj endokardiumának illetve kisebb mértékben epikardiumának elektrofiziológiai sajátságairól. A mélyebb rétegekből származó sejtek (M sejtek) elektrofiziológiai tulajdonságairól azonban viszonylag kevés adatunk van.

Az epikardiális és endokardiális felszín sejtjeiből elvezetett transzmembrán akciós potenciál regisztrátumok jelentős alaki eltérést mutatnak. Az epikardiális sejtek akciós potenciál amplitúdója általában kisebb, mint az endokardiális sejteké, mivel jóval kisebb a 0 fázisú (azaz a gyors depolarizáció alatt bekövetkező) „overshoot" (amikor a negatív membránpotenciál átmenetileg pozitívvá válik, overshoot = túllövés) (30, 39, 44). Az epikardiális akciós potenciált azonban egy szembetűnő „notch", azaz csomó jellemzi a 0 és 2 fázis (plató fázis) között, amely az endokardiális regisztrátumon nem látható (45). *(1. ábra)*. A „notch" következménye egy un. „spike and dome" (tüske és kupola) alakú akciós potenciál konfiguráció, mely legkifejezettebb a kutya (39), fokozatosan kevésbé a humán (19, 47), nyúl (26), és macska szív (34, 35) epikardiumon. A tengerimalac azon ritka fajok egyike, melynek epikardiális akciós potenciáljában hiányzik a „notch" (58). A „spike and dome" alakú epikardiális akciós potenciál élettani jelentősége még nem teljesen tisztázott, de az epikardiumtól az endokardium felé fokozatosan gyengülő "notch" következményének tekintik az EKG J-hullámát vagy J-pont elevációját (73). A J-hullám különösen jelentős a kutya elektrokardiogrammon, és ugyanezen fajban legkifejezettebb az epikardiális sejt akciós potenciálján a „notch".

M sejtek

Solberg és mtsai tettek először említést arról, hogy a mélyebb szívizom rétegekből származó sejtek elektrofiziológiai jellemzői különböznek a felszíni izomsejtekétől (60). Ezek a szerzők kutyaszívből izolált kamrai papilláris izom különböző transzmurális rétegeiből intracelluláris mikroelektród technika alkalmazásával elvezetett transzmembrán akciós potenciálokat tanulmányoztak. Leírták, hogy a mélyebb miokardiális sejtekben



1. ábra. Epikardiális sejtből (bal oldali ábra) és papilláris izomból (jobb oldali ábra) elvezetett transzmembrán akciós potenciál regisztrátumok. Ciklushossz: 500 ms. (Ref. 39)

(> 1 mm távolságra az endokardiális felszíntől) a nyugalmi potenciál negatívabb, az akciós potenciál amplitúdó magasabb, a depolarizáció maximális sebessége (V_{max}) pedig gyorsabb volt, mint a felszíni epikardiális és endokardiális sejtekben. Néhány mélyebb rétegből származó sejtben a felszíni sejtekénél jelentősen hosszabb időtartamú akciós potenciált találtak. A fenti tulajdonságok alapján ezen sejtek inkább az ingerületvezetésre specializálódott Purkinje rostokra hasonlítottak (15). A szubendokardiális Purkinje rostok a kutya szívizomzatban azonban nem hatolnak 2 mm-nél mélyebbre (61). Ezenkívül a mély miokardiális sejtek nem mutatták a Purkinje rostok egyik fontos jellegzetességét, a spontán diasztolés depolarizációt (15). Ezek a sejtek tehát nem lehetnek intramurális Purkinje sejtek. Solberg és mtsai vélekedése szerint ezen mély miokardiális sejtek a papilláris izom gyors aktiválódását facilitálnák, szerepük azonban továbbra is tisztázatlan maradt.

Mintegy 17 évvel később Sicouri és Antzelevitch egy sajátságos tulajdonságokkal rendelkező szívizomsejt populációt írt le a kutyaszív bal kamrai mély szubepikardiális régiójában, amelyeket M sejteknek neveztek el (51). Azóta egyre több tanulmány hangsúlyozza a kamrában fennálló heterogenitás fontosságát, különös jelentőséget tulajdonítva a kutya (9, 24, 42, 43, 51, 52, 53, 54, 57), tengeri malac (58), nyúl (68) és humán szív (19) kamrai M sejtjeire jellemző egyediségnek, mind elektrofiziológiai, mind farmakológiai érzékenység szempontjából. A pitvarokban ezidáig nincs tudomásunk ezen sejttípus előfordulásáról. Az M sejtekre jellemző akciós potenciálok az epikardiális felszíntől 1.5–5.2 mm távolságra elhelyezkedő szívizom rétegek sejtjeiből vezethetők el *(2. ábra)*.

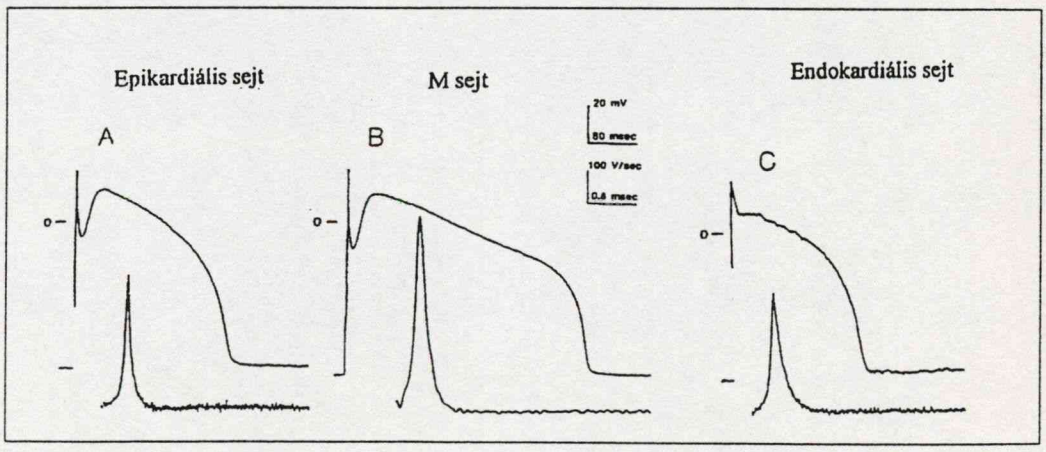
Elektrofiziológiai jellemzőik részben a munkaizomsejtekéhez, más szempontok alapján az ingerületvezető sejtekéhez hasonlítanak (6,54). Akciós potenciáljuk morfológiájára jellemző, noha valamivel kevésbé kifejezetten, az epikardiális sejtek elektromos válaszánál már említett „spike and dome" alakzat (2. ábra). Membránpotenciáljuk negatívabb, depolarizációjuk maximális sebessége (V_{max}) pedig nagyobb (≅ 330-500 V/s), mint az epikardiális és endokardiális sejteké, és megközelíti a Purkinje rostok ezen paraméterének nagyságát (400–1000 V/s). *(1. táblázat)* Spontán diasztolés

Az M sejtek elektrofiziológiai jellemzése: élettani, farmakológiai és klinikai jelentőségük

Kutyaszív bal kamrai epikardiális, M és endokardiális sejtjeiből, valamint Purkinje rostjaiból elvezetett akciós potenciálok paraméterei 1000 ms-os ingerlési ciklushossznál 1. TÁBLÁZAT

	Epikardium (n=29)	M sejt (n=37)	Endokardium (n=28)	Purkinje rost (n=20)
PR (mV)	-84,2±0,7	-86,5±0,8	-84.3±0,9	-89.6±0.9 *●▼
APA (mV)	101,3±1,2	108,4±1,5 ●	108,7±1,5 ●	124,6±1,7 *●▼
APD90 (ms)	222,1±5,3	258,7±4,5 ●	238,6±5,0	324,8±15,4 *●▼
Vmax (V/s)	154,5±6,6	284,0±17,5 ●	176,9±7,0 *	505,0±32,7 *●▼

Átlag ±S.E.; n=kísérletek száma; RP=nyugalmi potenciál; APA= akciós potenciál amplitúdó; APD_{90}=90 %-os repolarizáció; V_{max}=depolarizáció maximális sebessége. A szignifikanciát a variancia analízis nonparametrikus formájának Mann-Whitney, illetve Bonferroni szerinti eljárással való kapcsolásával határoztuk meg. * p<0,01 vs M sejt; ● p<0,01 vs epikardium; ▼ p<0,01 vs endokardium (Ref. 10)



2. ábra

Kutyaszív bal kamrai epikardiumából, M sejt régiójából és endokardiumából izolált szívizomsejtek akciós potenciálja és a depolarizáció maximális sebessége. Ciklushossz: 2000 ms. (Ref. 6)

depolarizációt azonban nem mutatnak, még az automáciát elősegítő körülmények között sem (alacsony extracelluláris K^+ koncentráció noradrenalin jelenlétében). Az elmondottakból következik, hogy az M sejtek több szempont alapján is a Solberg és mtsai. (60) által leírt mély miokardiális sejtekre emlékeztetnek, és Purkinje rost eredetük valószínűtlennek tűnik (54).

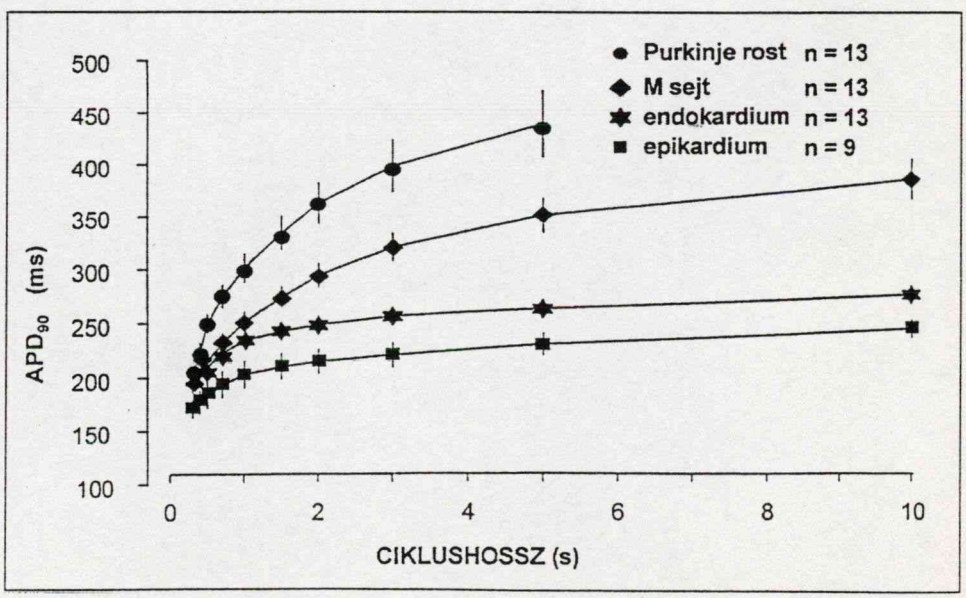
Az M sejtekből elvezetett transzmembrán akciós potenciál egyik legfontosabb jellegzetessége mind élettani, mind kórélettani és farmakológiai szempontból az a képesség, amelynek révén a kamrai szívizomzat másik két sejttípusához (endokardiális és epikardiális sejtek) viszonyítva aránytalanul nagy mértékű repolarizáció megnyúlással reagál mind a szívfrekvencia csökkenésére, mind az akciós potenciál időtartamát megnyújtó hatású gyógyszerekre (6, 9, 51). *(3., 4. ábra).*

Ezen jellegzetesség magyarázata az M sejtek illetve az endokardiális és epikardiális sejtek ioncsatornáiban megnyilvánuló különbségekre vezethető vissza. Mint ismeretes, a szívizomzat munkaizomsetjeinek repolarizációjáért, vagyis az eredeti negatív membránpotenciál visszaállásáért elsősorban a sejtből az extracelluláris tér felé történő káliummozgások felelősek, amelyek különböző K-csatornákon keresztül valósulnak meg (65).

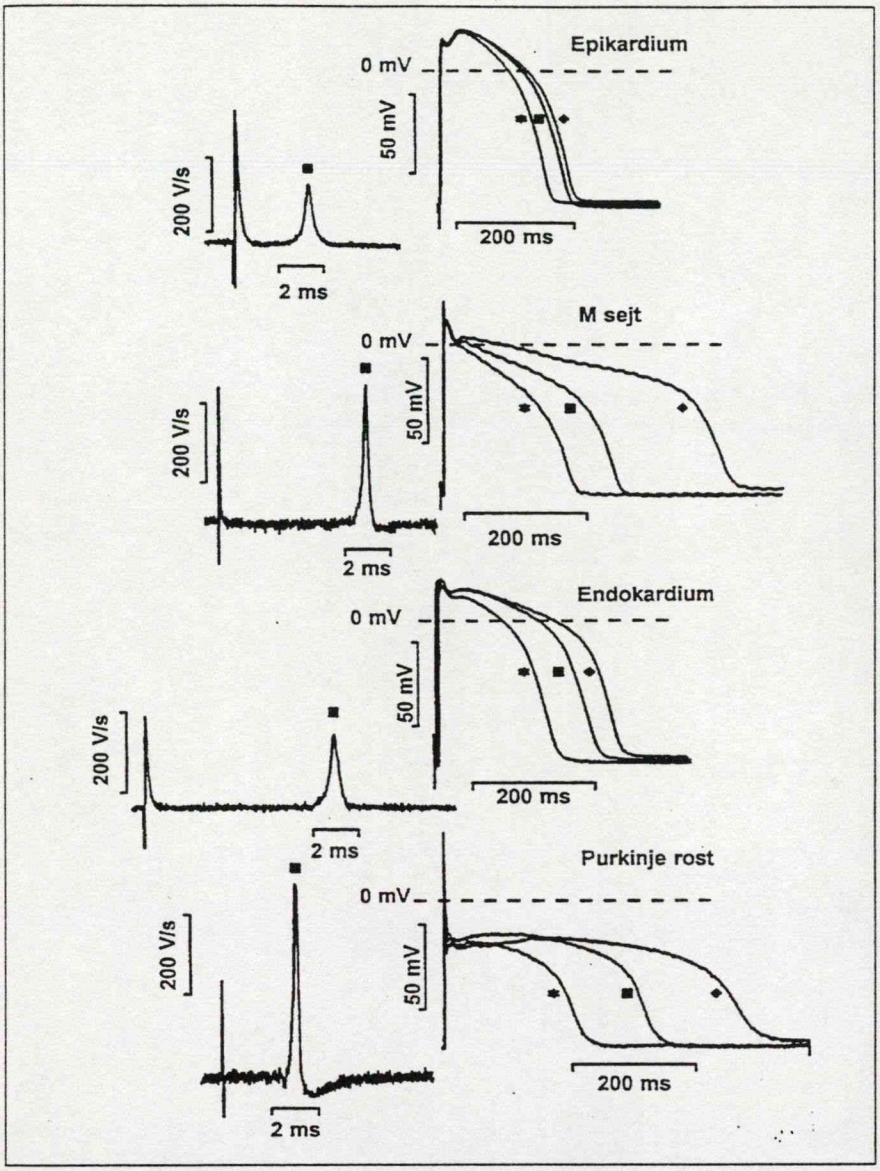
Ezek gátlása illetve gyengébb működése az akciós potenciál időtartam (APD) megnyúlását eredményezi. A gyógyszerhatások szempontjából legfontosabbnak tartott K-csatorna az un. késői egyenirányító (delayed rectifier) K-csatorna (I_K). Ezen ionáramnak két, egy gyorsan (I_{Kr}) és egy lassan aktiválódó (I_{Ks}) komponense ismeretes. Kutyaszív kamrai epikardiumából, M sejt régiójából, illetve endokardiumából izolált, különálló szívizomsejteket vizsgáló patch clamp és voltage clamp kísérletek (42) az M sejt akciós potenciál időtartamának (APD) drasztikus frekvenciafüggését részben az I_{Ks} ezen sejtekben viszonylag csekély csatornasűrűségével (43), részben pedig az akciós potenciál plató fázisa (2 fázis) alatt nyitva levő, lassan inaktiválódó (un. „window") reziduális Na-csatornák intenzívebb működésével hozták összefüggésbe (20). Az M sejtek ugyancsak kutyán in vivo módszerekkel történő azonosításáról beszámoló tanulmányok is napvilágot láttak (24, 66, 69).

FARMAKOLÓGIAI JELLEGZETESSÉGEK

Az előzőekben ismertetett elektrofiziológiai heterogenitás fontos következménye, hogy a különféle kamrai sejttípusokban a gyógyszerek eltérő – olykor akár ellentétes – hatást hoznak létre. Mivel az un. tranziens kifelé irányuló K-áram (I_{to} = transient outward current), mely a gyors 1 fázisú repolarizációért felelős, az epikardiális sejtekben kifejezett, az endokardiális preparátumokban azonban kevésbé jelentős, számos gyógyszercsoport merően különböző hatást eredményez e két szövetben. Az alábbi szerek APD rövidülést okoznak epikardiális sejtekben, míg az endokardiális preparátumokon megnyúlást hoznak létre: 1) I_{to} gátlók (39,40); 2) Na-csatorna gátlók (36,37); 3) neurotranszmitterek (pl. acetylcholin) (41); 4) magas extracelluláris Ca^{2+}-koncentráció (18) és 5) ATP-függő K-csatorna aktiválók (4,17). Az említett gyógyszercsoportok (az I_{to} gátlók kivételével) ellentétes irányú APD változásban megnyilvánuló hatása megszüntethető az ugyancsak I_{to} gátló 4-aminopyridin alkalmazásával. Tehát a tranziens kifelé irányuló K-áram (I_{to}) je-



3. ábra
90 %-os repolarizációnál mért akciós potenciál időtartam (APD90) frekvenciafüggése kutyaszív endokardiális, epikardiális és M sejtjeiben valamint Purkinje rostjaiban. Az abszcisszán a ciklushosszt, az ordinátán az APD90-et tüntettük fel. Az ábrán az átlagértékek mellett a ± standard error szerepel. (Ref. 10) Az ábráról leolvasható, hogy gyors szívfrekvencia esetén (rövid ciklushosszaknál), csökken a négy különböző sejttípus között fennálló repolarizációbeli különbség, míg a frekvencia lassulásával (hosszú ciklushosszaknál) az M sejtek APD-ingerlési frekvencia összefüggése az epi- illetve endokardiális sejtekénél jóval meredekebb lesz.



4. ábra. Kutyaszív bal kamrai epikardiális, endokardiális és M sejtjéből valamint Purkinje rostjából regisztrált transzmembrán akciós potenciálok (jobb oldali ábra) különböző ciklushosszúságú egyenletes ritmusú ingerlés idején: alap ciklushossz 300 ms (csillag), 1000 ms (négyzet) és 5000 ms (rombusz). A depolarizáció maximális sebessége (V_{max}) a különböző bal kamrai preparátumokban 1000 ms-os (négyzet) ciklushossz esetén (bal oldali ábra). (Ref. 10)

lentékenyen befolyásolja a kamrai felszíni sejtek farmakológiai válaszát azon szerekre, melyek megbontják a befelé (I_{Na} és I_{Ca}) és kifelé irányuló áramok (I_{to} és IK-ATP) között fennálló érzékeny egyensúlyt.

Az M sejtek sajátos – az előző két sejttípusétól merőben különböző – farmakológiai érzékenysége az e sejtekben gyengébb késői egyenirányító K-áram lassú komponensének (I_{Ks}) tulajdonítható (43). Ennek megfelelően az M sejtek mindenekelőtt abban különböznek az epikardium és endokardium sejtjeitől, hogy sokkal érzékenyebben reagálnak minden olyan szer hatására, mely akciós potenciál időtartam (APD) megnyúlást (elsősorban III. hatástípusú antiaritmiás szerek) illetve korai (early afterdepolarization = EAD) és késői utódepolarizációt (delayed

Az M sejtek elektrofiziológiai jellemzése: élettani, farmakológiai és klinikai jelentőségük

afterdepolarization = DAD) okoznak (8, 52, 53, 57). A korai utódepolarizációt okozó szerek a szívizomsejt akciós potenciál plató fázisa során a depolarizáló és repolarizáló áramok között fennálló egyensúlyt megbontva, a következő három fő mechanizmus révén okoznak triggerelt aktivitást: 1) repolarizáló K-áramok, elsősorban az I_{Kr} és I_{Ks}, csökkentése (I/A típusú szerek [kinidin, prokainamid] (53), III. osztályú szerek [d-sotalol, bretylium, clofilium, E-4031] (7, 57), egyes makrolid típusú antibiotikumok [erythromycin, spiramycin] (8, 62); 2) depolarizáló Ca-áram növelése [Bay K 8644] (7, 52) és 3) az un. „háttér" (window) Na-áram inaktivációjának késleltetése [aconitin, tengeri anemone toxin] (23). Az M sejtek korai utódepolarizációt kiváltó szerek iránti érzékenysége a Purkinje rostokéval vetekszik (16, 33, 75), mely tulajdonság a két sejttípus fiziológiás körülmények között is hosszabb APD-jével; ill. a frekvenciacsökkenésre és a késői egyenirányító K-áram gyors komponensének (I_{Kr}) blokkolására bekövetkező extrém APD megnyúlással magyarázható. A korai utódepolarizációt kiváltó mechanizmus azonban különbözőnek bizonyult a két settípusban. Kísérletes vizsgálatok ugyanis azt igazolták, hogy az M sejtekben képződött EAD a szarkoplazmatikus retikulumból történő kalcium felszabadulás gátlásával (ryanodine) APD rövidülés révén teljes mértékben megszüntethető, míg Purkinje rostokban ez a beavatkozás az EAD megszüntetése nélkül, további APD megnyúlást eredményez (13).

További farmakológiai eltérés az M és Purkinje sejtek között az α adrenerg receptor agonista szerekre (phenylephrin, methoxamin) bekövetkező ellentétes irányú APD változás (Purkinje rostokon megnyúlás, M sejteken rövidülés) (12).

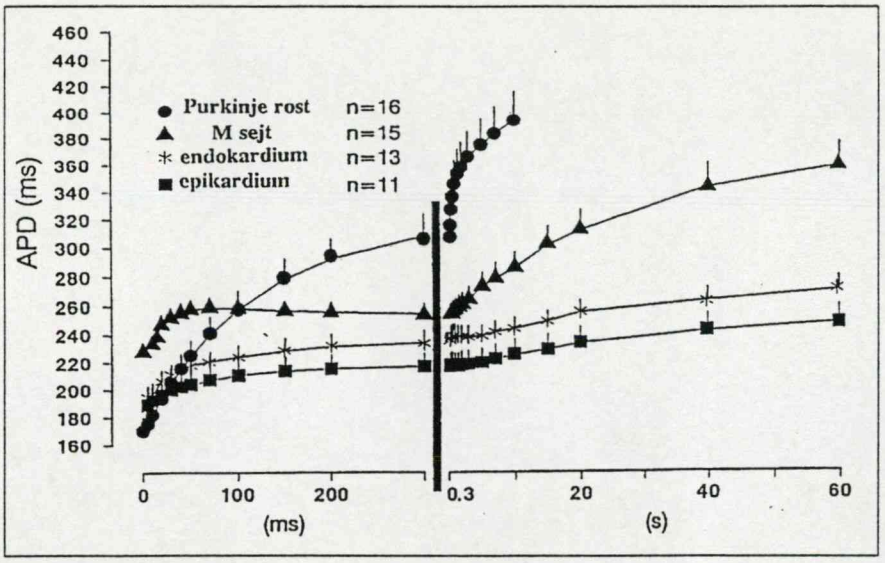
A kamrai sejttípusok eddigiekben említett szerekre adott eltérő válasza tovább fokozza a fiziológiás körülmények között is különböző hosszúságú APD-vel rendelkező szövetek által eredményezett transzmurális repolarizációs heterogenitást, azaz diszperziót a szívkamrákban. Jelenleg az amiodaron az egyetlen olyan antiaritmiás gyógyszer, amely valamennyi frekvenciatartományban csökkenteni látszik a transzmurális repolarizációs inhomogenitást oly módon, hogy a repolarizációs időt az endo- és epikardiális munkaizomzatban késlelteti, az M sejtekben nem okoz számottevő APD megnyúlást, a Purkinje rostokban pedig rövidíti azt (48,56,59).

AZ M SEJTEK ÉLETTANI FUNKCIÓJA

Az M sejtekről ezidáig nyert kísérleti eredmények alapján Antzelevitch a következő hipotézissel magyarázza ezen sejteknek a szívben fiziológiás ill. kóros állapotokban betöltött szerepét (2). Minthogy feltehetőleg a kamrák legnagyobb tömegét kitevő sejtpopulációról van szó (55), amely ezáltal döntő módon képviseli e szívüregek „valódi" munkaizomsejtjeit (46), az M sejtek valószínűleg a pumpafunkció hatékonyságának javítása céljából fejlődtek ki. Kiváltképpen igaz ez lassú frekvenciáknál, amikor a tartósabb depolarizáció hosszabb és erőteljesebb kontrakciókat tesz lehetővé. Az epi- és endokardium funkcionális elkülönülésére feltehetőleg azért volt szükség, hogy e rétegek az elektromos kapcsolódás révén mintegy gyengítsék az M sejt akciós potenciál extrém megnyúlását és utódepolarizáció létrejöttét, lehetséges szerepük tehát az M sejtek elektromos stabilizálása lenne. A teória értelmében az epikardium vagy endokardium egy szegmensének eltávolítása vagy infarktusa a QT-távolság megnövekedését eredményezi az M sejt APD megnyúlása következtében. Ez a mechanizmus lehet az alapja a hosszabb QT-távolságnak és megnövekedett QT-diszperziónak olyan non-Q-infarktuson átesett betegekben (az infarktust követő 2. és 3. napon), akikben az endokardium egy keskeny részlete nekrotizált (14, 73). A hipotézist igazolják azok a kísérletek is, melyekben az artériás perfúzió biztosítása mellett intakt állapotban tartott kamrából történő endokardium eltávolítás az M sejt APD és a QT-távolság megnyúlását, következésképpen a transzmurális repolarizációs diszperzió fokozódását okozta (8). Az infarktus ezen QT-szakaszt illetve QT-diszperziót átmenetileg növelő hatása feltehetőleg még határozottabban jelentkezik III. osztályú antiaritmiás gyógyszerekkel kezelt, illetőleg a hosszú QT-szindróma veleszületett vagy szerzett formájában szenvedő betegeknél (8), amely kombináció mind monomorf, mind polimorf kamrai aritmia fenntartására alkalmas aritmogén szubsztrátumot sejtet.

REPOLARIZÁCIÓS DISZPERZIÓ ÉS AZ M SEJTEK SZEREPE ARITMIÁKBAN

Amint azt már a korábbiakban említettük, a kamrafali sejtek főként repolarizációjuk karakterében térnek el egymástól. Az M sejtek a Purkinje rostokéhoz hasonló, nagy mértékű APD megnyúlással reagálnak a szívfrekvencia lassulására, mely jelenség önmagában is, de a repolarizációs időt megnövelő gyógyszerek hatására még kifejezettebb repolarizáció és refrakteritásbeli diszperziót eredményez mind az M sejtek és a kamrafali munkaizomsejtek másik két típusa (51), mind pedig a kamraizomzat és a His-Purkinje rendszer (3) között. (3., 4. ábra) Az M sejtek jóval meredekebb APD-ingerlési frekvencia összefüggése különböző ciklushosszúságú egyenletes ritmusú ingerlés (3. ábra) és tesztimpulzusok segítségével egyre növekvő diasztolés intervallummal kiváltott extraszisztolék akciós potenciáljainak időtartamait megmérve (restitúció) egyaránt tapasztalható *(5. ábra)* (10,51). A multicelluláris preparátumokban mért repolarizációs időbeli különbségek kvantitatíve megfeleltek a kamrafal három rétegéből enzimes emésztéssel izolált, különálló szívizomsejtekben talált adatoknak (42, 43, 44). Artériás perfúzió biztosítása mellett, intakt állapotban tartott balkamrafali preparátum elvezetéseit vizsgálva azonban – amikor a három sejttípus között megtartott az intakt állapotra jellemző erős elektrotónusos kapcsolódás – a repolarizáció diszperziója nagy mértékű csökkenést mutatott, mivel a különböző sejtek közötti elektrotónusos befolyás az M sejtek APD-jét saját, un. „intrinsic" értéke alá rövidíti, míg az epi- és endokardiális sejtek akciós potenciálját a rájuk jellemző „intrinsic"érték fölé nyújtja (8, 72). Ezen kísérleti modellben megfigyelthez minőségében hasonló diszperziót írtak le kutyaszívben in vivo körülmények között is, a transzmurális régióról regisztrált monofázisos akciós potenciálok [MAP] (69, 70), illetve unipoláris elvezetések segítségével számított aktivációs/repolarizációs idő [ARI] mérésével (24). Az in vivo modelleknél fontos moduláló szerepet tulajdonítanak az alkalmazott anesztézia típusának. Kutyában az elülső balkamrafalon MAP-technikával mért repolarizációs diszperzió számottevően kisebbnek bizonyult Napentobarbitállal, mint halothannal altatott állatokban (2), és I_{Kr} blokkoló szer infundálásakor ez a különbség tovább fokozódott (69, 70). Elképzelhető, hogy ez a metodikabeli különbség felelős azért,



5. ábra A teszt akciós potenciál időtartam (APD) és a diasztolés intervallum összefüggése kutyaszív bal kamrai epikardiális, endokardiális és M sejtekben, valamint Purkinje rostokban. Ciklushossz: 1000 ms. Az abszcisszán a diasztolés intervallumot (azaz a teszt akciós potenciál felszálló szára és az ezt közvetlenül megelőző alapritmusú akciós potenciál 90 %-os repolarizációja között eltelt időt), az ordinátán a teszt akciós potenciál (azaz extraszisztole) időtartamát tüntettük fel. Az ábrán az átlag mellett a ± standard errort is jelöltük. A restitúciós görbe kezdeti szakaszán az időskálát felnagyítottuk annak érdekében, hogy a korai extraszisztolék APD értékeit jobban elkülönítve ábrázolhassuk. (Ref. 10) Az ábráról leolvasható, hogy az M sejtekben kiváltott extraszisztolék akciós potenciáljaink időtartama egyaránt hosszabb az epi- illetve endokardiális sejtek APD-jénél mind rövid, mind fiziológiás és hosszú diasztolés intervallumoknál, és ez a különbség az ingerlési frekvencia csökkenésével (hosszú DI-k) egyre fokozódik.

hogy Rosen és mtsai. illetve Friegang és mtsai. in vivo modellen nem találtak jelentős repolarizációs grádienst a balkamrafalban (9, 27), míg mások kimutatták azt (24, 70).

Az elmondottakból következik, hogy noha az M sejtek pontos funkciója még nem kellőképpen tisztázott, a repolarizáció általuk okozott inhomogenitása fontos kiindulópontja lehet számos reentry mechanizmusú aritmiának. Figyelembe véve azt, hogy a szabad balkamrafal legalább 30-40 %-át kitevő sejtpopulációról van szó (55), az aritmogenezisben különös jelentőségű szerepük valószínűsíthető. A mai álláspont alapján az M sejt régió APD-jének tetemes megnyúlási készsége áll a hosszú QT-szindróma (LQTS) hátterében, és ez jelentékeny mértékben hozzájárul a QT szakasz megnyúlásához, kóros U-hullám (T2) valamint kaotikus, torsade de pointes típusú polimorf kamrai tachycardia (TdP VT) kialakulásához (5). A hosszú QT/QTU-szindróma talaján fellépő, bizarr QRS-morfológiával rendelkező TdP VT-t a legújabb kísérletes vizsgálatokkal igazolt szemlélet szerint a Purkinje és M sejtekben keletkező korai utódepolarizáció (EAD) indítja el és intramurális reentry tartja fenn (24,49). Ismeretes, hogy a Purkinje és M sejtek akciós potenciáljai több vonatkozásban hasonlítanak egymáshoz: 1) APD-jük hosszabb az endo- és epikardiális munkaizomsejtekénél; 2) erős tranziens kifelé irányuló K^+-árammal (I_{to}) rendelkeznek, ami az akciós potenciál kezdeti repolarizációs fázisának alakját karakterisztikussá („spike and dome") teszi; 3) APD-jük hypokalaemia, bradycardia ill. pulzus kimaradás és bizonyos gyógyszerek hatására nagymértékben megnyúlik (8, 24, 48,7 4). Ezen két – frekvenciacsökkenésre és a késői

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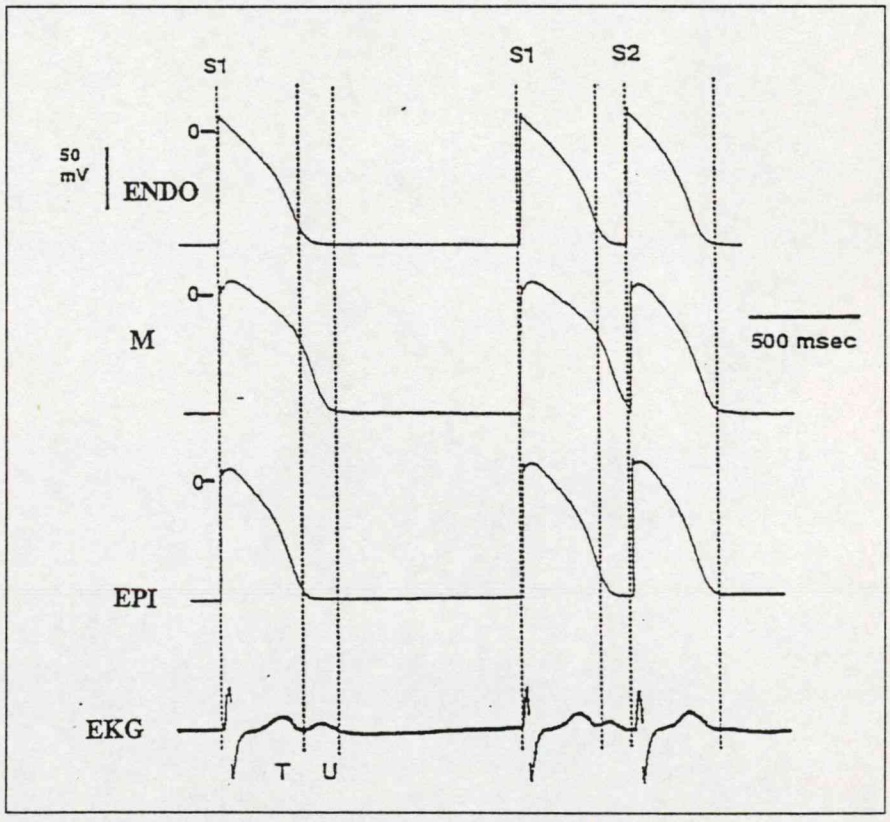
egyenirányító K^+-áram gyors komponensének (I_{Kr}) blokkjára drasztikus APD megnyúlással reagáló –sejttípus elektrofiziológiai jellegzetességeinek ismeretében csaknem bizonyosra vehető, hogy a szerzett (bradycardia-dependens) hosszú QT-szindrómában fellépő EAD a Purkinje és M sejtekben keletkezik (8). El-Sherif és mtsai. a szerzett hosszú QT-szindróma egy in vivo kutya-modelljében háromdimenziós aktivációs/repolarizációs térképezéssel azt bizonyították, hogy a Purkinje rostokból kiinduló fokális aktivitás elsősorban azokban a környező miokardium régiókban indukál circus movement reentry-t, amelyekben a kamrai repolarizáció intramurális diszperziója nagy (24). Ezen fokozott intramurális repolarizációs heterogenitás létrejöttében nyilvánvalóan alapvető szerepe van a Purkinje rostokhoz számos szempontból hasonlóan viselkedő, ám a kamrák azoknál jóval tekintélyesebb tömegét (30-40 %) kitevő M sejteknek. Az újonnan kifejlesztett szelektív I_{Kr} blokkoló antaritmiás vegyületek (d-sotalol, dofetilid, almokalant, sematilid, GLG-V13) elfogadhatatlanul magas proaritmiás („torsadogén") aktivitása is részben arra vezethető vissza, hogy APD-t megnyújtó hatásuk számottevően erősebb a Purkinje és M sejtekben, s jóval kisebb mértékű az endo- és epikardiális munkaizomzatban, ami összességében a transzmurális repolarizációs inhomogenitás és intramurális reentryhajlam növekedéséhez vezet (1, 25, 64). Az elmondottak alapján érthető, hogy miért előnytelen klinikai szempontból a specifikus I_{Kr}

gátló gyógyszerek azon tulajdonsága is, amelynek révén az általuk előidézett APD és refrakteritás megnyúlás mind a Purkinje, mind az M sejtekben egyaránt fordítottan frekvenciafüggő (reverse use-dependent) módon jelentkezik, az az sinus-ritmus és bradycardia idején túlságosan erős, tachycardia, tachyaritmia fennállásakor viszont – noha a refrakteritást megnyújtó hatásra éppen ilyenkor lenne szükség – kevésbé érvényesül (74).

AZ M SEJTEK SZEREPE AZ EKG U-HULLÁMÁBAN

Az M sejtek elektrofarmakológiájának megismerése segítséget nyújthat egy tisztázatlan eredetű EKG jelenség, nevezetesen az U-hullám keletkezésének megértéséhez. Egészséges emberek 40%-ának EKG-ján megtalálható a T-hullámot követő, attól definitíve elkülönülő U-hullám, amelynek jelentősége egyelőre még ismeretlen (32). A számos teória közül, mely ezen EKG hullám eredete magyarázatául szolgál, az utóbbi időkig a következő tűnt a legelfogadottabbnak. Ezen hipotézis szerint a kamraizomzatban létrejövőhöz képest késve, és annál sokkal elhúzódóbban lezajló szubendokardiális Purkinje rendszerbeli repolarizáció lenne felelős az U-hullám generálásáért (67). A kamrákban szabadon futó Purkinje rostok (free running Purkinje fibers) akciós potenciálja valóban számottevően hosszabb, mint a munkaizomrostoké és időben egybeesik az U-hullám megjelenésével. A balkamra üregét sűrűn behálózó szubendokardiális Purkinje rost hálózat APD-je azonban csak alig hosszabb a kamraizomrostokénál. Ez az egyik oka annak, amiért a Purkinje rostok szerepe az U-hullám keletkezésében nem valószínű. A másik kétséget ébresztő tényező a Purkinje rost rendszer kicsiny tömege, hogy ti. képes-e ez önmagában akkora áramot indukálni, hogy annak eredménye a felszíni EKG-n észrevehető feszültséghullám legyen (11). A diszkrepancia még határozottabban jelentkezik az elektrolitzavarban szenvedő betegeknél regisztrált un. „óriás" U-hullám észlelésekor, amelynek mérete rendszerint a T-hulláméhak többszöröse (31, 50). A közelmúltban született elképzelés szerint az U-hullám a balkamra M sejt régióját jellemző elhúzódó repolarizáció megnyilvánulása (7, 19, 38). Minthogy az M sejtek APD-je jelentősen hosszabb, mint az epikardiális és endokardiális sejteké, késleltetik a kamrafal nekik megfelelő területén a repolarizáció lezajlását, aminek következménye azon áram keletkezése, amely az EKG-n az U-hullámot eredményezi *(6. ábra)*. Az M sejt hipotézist számos tényező támasztja alá. 1) Az M sejtek a balkamrafal tekintélyes hányadát (30-40 %) alkotják, tehát elegendő tömegűnek tekinthetők ahhoz, hogy a felszíni EKG-n észrevehető feszültséghullámot generáljanak. 2) Az M sejtek főként lassú szívfrekvenciáknál elhúzódó repolarizációja (51, 54) időben megfelel az U-hullám megjelenésének (19). 3) Az M sejtek frekvenciafüggő repolarizációja összhangban áll az U-hullám frekvenciafüggésével. Gyors szívfrekvencia vagy korai extraszisztolék esetén csökken az M sejtek illetve epi- és endokardiális sejtek között fennálló repolarizációs diszperzió (3., 4. ábra), mely körülmények egyúttal az U-hullám csökkenését vagy megszűnését is eredmé-



6. ábra. Az M sejtek elhúzódó repolarizációja, mint az U-hullám alapja. Az ábrán humán transzmurális kamracsík epikardiális (EPI), endokardiális (ENDO) és M sejtjeiből elvezetett akciós potenciálok láthatók az EKG-val egybevetve, egy 85 éves betegen. A preparátum ingerlési ciklushossza 2000 ms, ami hasonló az EKG-n látható junkcionális ritmus frekvenciájához. Az endokardiális és M sejt repolarizáció végénél húzott függőleges vonalak a kamrai preparátum repolarizációjának diszperzióját szemléltetik, valamint annak összefüggését az U-hullám megjelenésével. Az S1-S2 = 500 ms kapcsolási idővel kiváltott extraszisztole mind a repolarizációs diszperzió, mind az U-hullám megszűnését eredményezi. (Ref. 19)

nyezik (6. ábra). Ezzel ellenkezőleg, lassú szívfrekvencia tetemesen megnyújtja az M sejtek akciós potenciálját és egyidejűleg az U-hullám amplitudóját is növeli. Érdekes megemlíteni, hogy Einthoven (az U-hullámot is ő írta le először és az elnevezés is tőle származik) eredeti feltételezése szerint az U-hullám olyan áramok következménye, amelyeket egy késői repolarizációval rendelkező szívizomterület generál (21, 22). Jelenleg úgy tűnik, hogy ezt a területet az M sejtek rétege képviseli.

KÖVETKEZTETÉS, PERSPEKTÍVÁK

A teljesség igénye nélkül számba vett nemzetközi tanulmányok, és a még limitált számú saját megfigyelés eredményei egyértelműen igazolták az elektrofiziológiai és farmakológiai tulajdonságaiban egyedülálló M sejtek jelenlétét a kutyaszív mély szubepikardiális régiójában. Továbbra is várat azonban magára a kamrafal különböző szövet- és sejttípusait minden részletében jellemző elektrofiziológiai és farmakológiai profil [pl. regionális (bázis, apex, szeptum, kifolyó traktus) és életkorfüggő sajátságok] meghatározása. A szívkamrák elektromos inhomogenitására irányuló további kutatások során tett megfigyelések új fejezetet nyithatnak meg a ritmuszavarok patomechanizmusának és az antiaritmiás gyógyszerek hatásmódjának megértésében, s ezáltal az aritmiák jelenleginél definitívebb, kevésbé empírikus terápiás megközelítésére nyílna lehetőség.

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Pharmacological Modification of the Dispersion of Repolarization in the Heart: Importance of the M Cells

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Summary. **Several in vitro and in vivo investigations have provided data supporting the existence of M cells in the deep subepicardial layers of the ventricles in a number of species. Characterized by unique electrophysiological and pharmacological features, this population of cells is regarded to have a significant role in creating dispersion of repolarization in the ventricular wall and thus contribute importantly to arrhythmogenesis, in particular to intramural reentry and triggered activity. Focusing on M cells, the authors summarize recent findings and concepts concerning the pharmacological heterogeneity of different cell and tissue types found within the ventricles and explore how these differences may contribute to electrocardiographic manifestations. On the basis of literarary data and of their own results they conclude that studying the electrical and pharmacological inhomogeneity within the ventricular wall may provide a better understanding of the pathophysiological processes that give rise to cardiac rhythm disturbances and the mechanisms by which antiarrhythmic agents act to suppress and in some cases aggravate arrhythmias.**

Key Words. **M cells, pharmacology, action potential duration, rate dependence, dispersion of repolarization, arrhythmia**

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 fibres, which are specialized for impulse conduction. Data obtained from endocardium, however, 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 [1,2] 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. Several previous investigations have highlighted the electrophysiological distinctions between the ventricular endocardium, the epicardium, and the Purkinje fibres, demonstrating different, and sometimes even opposite responses of these cell types to pharmacologic agents and pathophysiologic states in a number of species [3–10]. Nevertheless, these data failed to attract sufficient attention until the electrophysiological characterization of the cells in the midmyocardial layers of the ventricular myocardium. 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 [11], and found to have electrophysiological features intermediate between those of the myocardial and Purkinje cells (Table 1). It was also reported that they differ in pharmacological responsiveness from those of either the epicardium or the endocardium [12–16].

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. In this review, we discuss the pharmacological influence of the dispersion of repolarization in the ventricle at the cellular level, focusing on the data that have recently become available on M cells.

Electrophysiology of M Cells

Sicouri and Antzelevitch characterized the electrophysiological properties of a subpopulation of cells in the deep subepicardial to midmyocardial layers of the canine ventricular free wall [11], these cells have been termed M cells. M cells have also been found in the

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Table 1. 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; RP = resting potential; APA = amplitude of the action potential; APD_{90} = action potential duration at 90% repolarization; V_{max} = maximal rate of rise of the action potential upstroke. 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. From Baláti et al. 1998, with permission.

deep layers of the septum, papillary muscles and trabeculae [17]. An increasing number of studies have subsequently attributed particular importance to the unique electrophysiological characteristics and pharmacological responsiveness of M cells. M cells have likewise been described in guinea pig [18], rabbit [19] and human ventricles [20]. One study did not verify the existence of these cells in the pig [21], but their absence may have been related to maturity rather than to species differences [22]. M cells have the ability to prolong the action potential duration (APD) disproportionately relative to the endocardial or epicardial cells in response to a slowing of the stimulation rate and/or to agents that lengthen the APD. At moderate to fast stimulation rates, the only discernible difference as concerns cells spanning the ventricular free wall is in the spike-and-dome morphology of the action potential [1]; prominent in the epicardium, the notch gradually diminishes across the wall to the endocardial surface (Fig. 1). With progressive slowing of the stimulation rate, however, another important distinction becomes increasingly apparent; M cells exhibit a much more prolonged APD than those of cells in the epicardial and endocardial regions of the wall (Figs. 2 and 3). This characteristic feature of M cells is expressed in isolated cells [23], in multicellular isolated preparations [11,24], in arterially perfused wedge preparations [25] and in the intact whole heart as well [26]. The APD-rate relationships observed in M cells are similar to those in Purkinje fibres (Figs. 2 and 3), which were extensively studied earlier. In addition to the marked rate-dependent change of APD in M cells, the high value of the maximum rate of rise of their action potential upstroke (V_{max}) resembles also that observed in Purkinje fibres [11]. However, as an important difference from Purkinje fibres, M cells exhibit no phase 4 depolarization, not even in the presence of catecholamines and low potassium concentration, and their plateau develops at

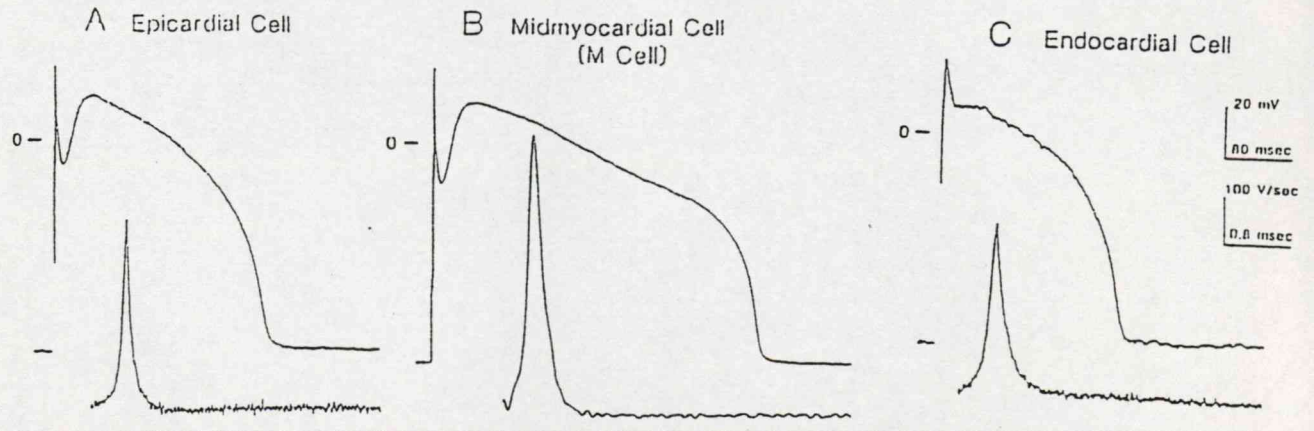

Fig. 1. Action potentials recorded in myocytes enzymatically dissociated from epicardial (panel A), midmyocardial (M cell; panel B), and endocardial (panel C) tissues obtained from the free wall of the left ventricle of canine hearts. Each panel (A-C) shows tracings of the action potential and the maximum rate of rise of the action potential upstroke, recorded using a standard microelectrode at a basic cycle length (BCL) of 2000 ms. $[Ca^{2+}]_o$ = 2 mM; $[K^+]_o$ = 6 mM. From Antzelevitch et al. 1991, with permission.

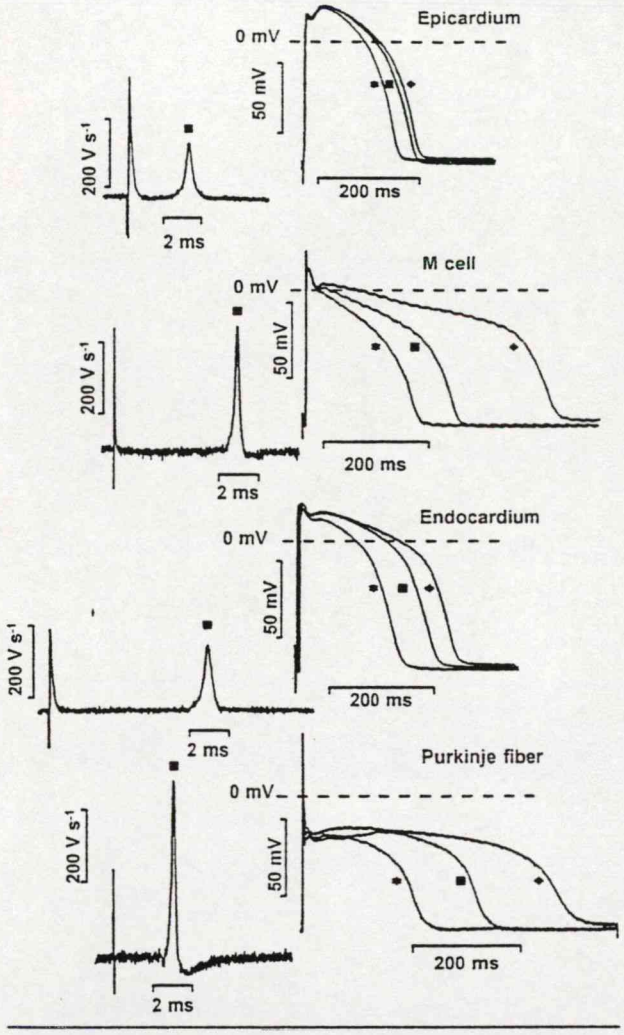

Fig. 2. *Transmembrane action potentials (right panel) recorded under steady-state conditions at basic cycle lengths of 300 (star), 1,000 (square) and 5,000 ms (diamond) from epicardium, midmyocardium (M cell), endocardium and Purkinje fibers isolated from the canine left ventricle. The maximal rate of rise of the action potential upstroke (V_{max}) (left panel) recorded from the respective regions of the left ventricle at a basic cycle length of 1,000 ms (square). From Baláti et al. 1998, with permission.*

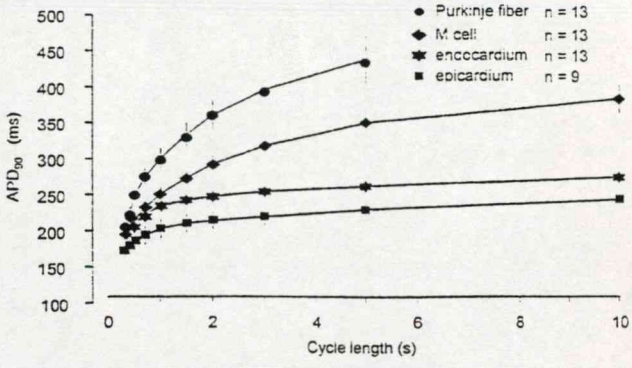

Fig. 3. *Rate dependence of action potential duration measured at 90% repolarization (APD_{90}) in epicardial cells, M cells, endocardial cells and Purkinje fibres of the canine left ventricle. Mean ± SEM values are shown for basic cycle lengths (BCLs) of 300, 400, 500, 700, 1000, 1500, 2000, 3000, 5000 and 10000 ms. From Baláti et al. 1998, with permission.*

more positive potentials [24] (Fig. 2). It may be concluded therefore that M cells display characteristics common to both working myocardial cells (spike-and-dome morphology, the absence of phase 4 depolarization, and a positive plateau phase) and Purkinje fibres (higher V_{max} and steeper APD-rate relation). The ionic basis for the unique repolarization feature of M cells is supposed to include the presence of a smaller density of the slowly activating component of the delayed rectifier potassium current (I_{Ks}) [27] and also a larger density of the window (or slowly inactivating) sodium current [28].

The greater prolongation of APD in M cells and also in Purkinje fibres at slow rate (Figs. 2 and 3) and after the application of certain drugs could enhance the dispersion of repolarization and refractoriness within the ventricle [29]. This heterogeneity may provide an important substrate for early afterdepolarization (EAD) related reentrant arrhythmias, including torsade de pointes (TdP). Although M cells are better coupled than Purkinje fibres, it has been estimated that they comprise at least 40% of the total ventricular myocardial mass [17], and their possible role in arrhythmogenesis is therefore of especially great importance.

It has been suggested that, since M cells may be the most abundant cell population in the ventricles and may largely represent the working myocardium [22], 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 [22]. The 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 the epicardium or endocardium would be expected to lead to increases in the QT interval and QT dispersion secondary to a prolongation of the M cell APD [30,31]. In patients treated with drugs exhibiting Class III antiarrhythmic action or in those having a congenital or acquired long QT syndrome, these transient increases in QT and QT dispersion might be even more amplified [32], and may result in monomorphic and polymorphic arrhythmias.

Pharmacological Responses

With regard to the marked electrophysiological heterogeneity exhibited by the four types of cells in the

ventricle (Table 1; Figs. 2 and 3), it may be expected that they will display different responses to a wide variety of pharmacological agents. The various combinations of the different K^+ currents and the different magnitudes of the inward sodium and calcium currents in each cell type lead to major differences in the responses to drugs in the epicardial, M and endocardial cells and the Purkinje fibres. The transient outward current (I_{to}) is carried primarily by K ions. It has long been known that I_{to} is well developed in the Purkinje fibres, but the possibility that it is more prevalent in specific layers of the ventricles was first suggested by Litovsky and Antzelevitch [4]. They demonstrated that 4-aminopyridine abolished the action potential notch (which is responsible for the spike-and-dome morphology) in canine epicardial tissue, but had little effect on the endocardial response. Consequently, the epicardial and endocardial action potentials became very similar after I_{to} inhibition. Their hypothesis was confirmed by measurements of the levels of I_{to} in canine epicardial and endocardial myocytes by means of whole-cell patch clamp techniques [23]. The results implied that the prominent action potential notch in the epicardium was due to a large I_{to} that was lacking in the endocardium. The presence of a large I_{to} in the epicardium, but not in the endocardium, results in the different, and often opposite, responses of these two cell types to different drugs. An interesting hypothesis was put forward by Rose et al. [33] on the basis of their experimental observations. In rabbit ventricular myocytes, a significant APD prolongation was found after the blocking of I_{to}, while a shortening of APD was found after current pulses which made the plateau phase more negative. It was concluded that at less positive voltages the rapidly activating component of the delayed rectifier potassium current (I_{Kr}) recovers more from its inactivation and contributes more to repolarization. It is tempting to speculate that in the Purkinje fibres, where the plateau phase is at a relatively negative potential, the effect of I_{Kr} block in lengthening repolarization is always large, which may be explained by similar mechanisms.

The unique pharmacological responsiveness of M cells is supposed to be due to the reduced density of the delayed rectifier potassium current (I_K) in these cells. Liu and Antzelevitch [27] compared I_K in the epicardial, M and endocardial cells of the canine ventricle and found that the amplitude of the current in the M cells was considerably lower than that in either the epicardial or the endocardial cells. After characterization of the two components of I_K in the different cell types on the basis of E-4031 (I_{Kr} blocker) sensitivity, they found that the I_{Kr} density was similar in the three cell types, whereas I_{Ks} was significantly smaller in the M cells than in the epicardial and endocardial cells. In concordance with the above, M cells differ primarily from the other working myocardial cell types in their ability to exhibit marked action potential prolongation, EADs and triggered activity in response to a diverse group of agents.

In contrast with M cells, it is difficult to induce EADs in epicardial or endocardial cells by any means.

The sensitivity of M cells to agents that induce EADs is similar to that observed in Purkinje fibres [34–36]. This can be explained by the longer APD of these two cell types, and especially by the dramatic prolongation of APD in bradycardia and after the inhibition of I_{Kr}. However, the mechanisms underlying the development of EADs in the two cell types appear to be different. It has been shown that in M cells the EADs induced by the selective I_{Kr} blocker E-4031 were suppressed by ryanodine (0.1–1.0 μM), an agent known to inhibit calcium release from the sarcoplasmic reticulum, with a concomitant abbreviation of APD at all rates. The resting membrane potential of M cells was not affected by ryanodine. In contrast, ryanodine did not suppress EADs in the Purkinje fibres, but instead caused a prolongation of the APD and depolarization of the resting membrane potential [37]. It was shown earlier that EAD elicited in the Purkinje fibres was suppressed by the calcium channel blocker verapamil [38]. This latter finding may relate to the observation that the plateau phase of the Purkinje fibres often develops at relatively negative potentials, where reactivation of the calcium current is possible. These findings suggest that EADs induced in M cells are particularly sensitive to changes in intracellular calcium levels, whereas EADs elicited in the Purkinje fibres are less sensitive. Since the plateau phase of the M cells is usually more positive than 0 mV, reactivation of the calcium current as a cause of EAD is not to be expected.

Agents can induce EADs by altering the balance of depolarizing and repolarizing currents during the plateau phase of the cardiac action potential [39]. This occurs via one of the following four mechanisms: (1) reductions in repolarizing K^+ currents, primarily in I_{Kr} and I_{Ks} (eg. Class I/A antiarrhythmic drugs [quinidine and procainamide] [13], Class III antiarrhythmic drugs [d-sotalol, bretylium, clofilium and E-4031] [2,14], some macrolide type antibiotics [erythromycin and spiramycin] [32,40]; (2) an increase in Ca^{2+} current [Bay K 8644] [2,12]; (3) a delay in Na^+ current inactivation [aconitine, sea anemone toxins and veratrine] [41]; and (4) an increase in the outward mode of the Na^+/Ca^{2+} exchanger current, most probably due to a calcium overload [42].

A) Reduction in repolarizing K^+ currents, as the primary cause for transmural dispersion of repolarization (TDR) in the ventricular wall

It was mentioned above that one of the major causes of the dramatic prolongation of the M cell and Purkinje fibre action potential and EAD formation is their unique response to different agents which act to re-

duce I_K, one of the main currents controlling repolarization in the mammalian heart.

D-sotalol has been shown to be effective in a variety of supraventricular and ventricular arrhythmias [43–45]. The antiarrhythmic effect of this drug is attributed to its Class III characteristics, which are due to the blockade of I_{Kr} [46], resulting in a prolongation of the APD and the effective refractory period in the atrium, ventricle and sinus node of different species in *in vitro* studies, and the epicardial and endocardial monophasic action potential (MAP) duration in anesthetized dogs [47] and the endocardial MAP in humans [48]. Despite its favourable antiarrhythmic action, d-sotalol can also be proarrhythmic: a recent clinical trial that evaluated d-sotalol in patients after myocardial infarction (SWORD) [49] had to be prematurely interrupted because of the excess mortality in the treated group. Sicouri et al. [14] tested the hypothesis that the M cells are the primary target for the Class III actions of d-sotalol in the canine ventricular myocardium and may contribute to its proarrhythmic effects [14]. They found that d-sotalol prolonged the APD in all 3 myocardial cell types. However, this effect was much more pronounced in the M cells than in the epicardial or endocardial cells, especially at the slower rate of stimulation; and as a consequence, it produced an increase in the TDR. They also evaluated the effects of d-sotalol on the characteristics of the restitution of the action potential in the different cell types. Under control conditions, the restitution curve of M cells is displaced upward relative to those of the epicardium and endocardium [11,24]. d-Sotalol induced an upward shift of the 3 curves, which was more accentuated in M cells. These results demonstrated a preferential effect of d-sotalol in prolonging the action potential of M cells with respect to that of epicardial or endocardial cells under both steady-state and non-steady-state stimulation conditions. EADs were observed in M cells, but not in epicardial or endocardial peparations following exposure to 100 μM d-sotalol and after prolongation of the basic cycle length (BCL) of the stimulation from 800 ms to 8000 ms. The effect of d-sotalol in producing EADs in M cells was similar to that previously observed in Purkinje fibres exposed to the same concentration of the drug [50].

In common with most drugs with Class III actions, d-sotalol manifests its greatest effect at slower rates and much less effect at faster rates (reverse use-dependence). This feature is especially noticeable in M cells and Purkinje fibres, due to the preferential effect of these agents on those tissues [2] and it also leads to a marked increase in the TDR, setting the stage for possible reentrant arrhythmias and TdP.

Although marked action potential prolongation, EAD and triggered activity were generally observed at slow rates of stimulation (BCL > 1000 ms) under steady-state conditions, a sudden acceleration of the rate could induce a paradoxical transient prolongation of the M cell action potential, as illustrated in Figure 4. Panel A shows the APD changes in time following an

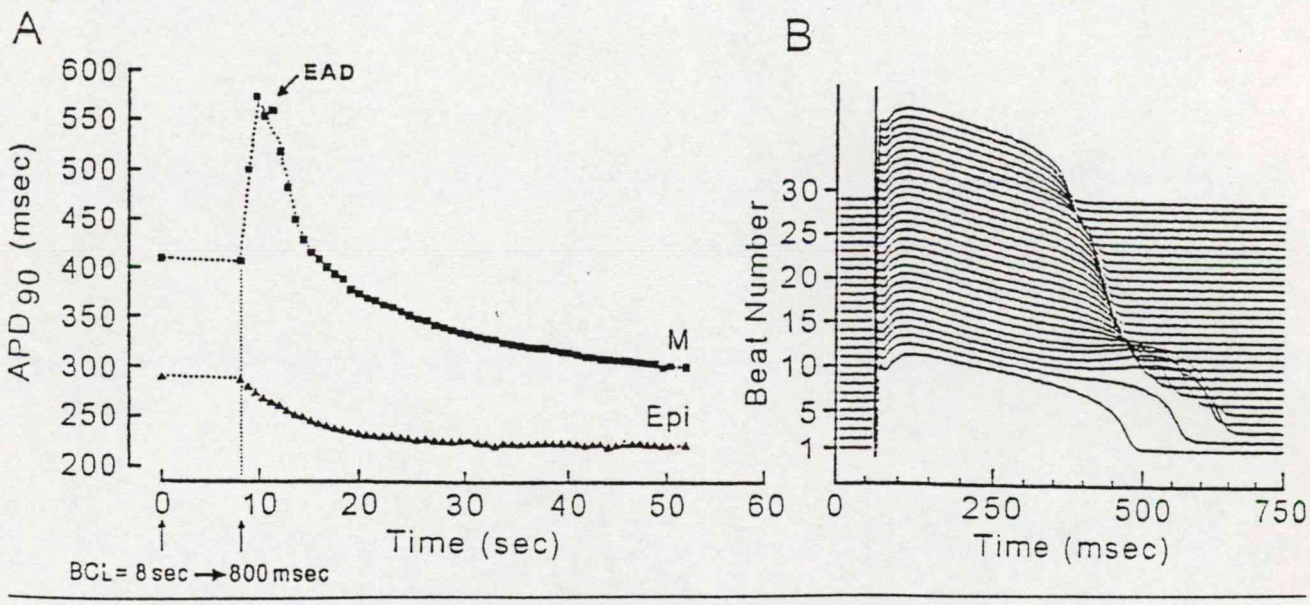

Fig. 4. *d-Sotalol-induced action potential duration (APD) prolongation and early afterdepolarization (EAD) in M, but not in epicardial (Epi) tissues, following acceleration of the stimulation rate. (A) Graphs displaying time courses of APD changes in M cells and Epi preparations following an abrupt acceleration of the stimulation frequency from a BCL of 8000 ms (beats 1 and 2) to a BCL of 800 ms (subsequent beats) 30 minutes after exposure to 100 μM d-sotalol. (B) Transmembrane activity recorded from the M cell preparation. Beat 1 (lower action potential) at a BCL of 8000 ms; subsequent beats at a BCL of 800 ms. APD prolongation and EAD are apparent only in M cell.* $[K^+]_o$ *= 4 mM. From Sicouri et al. 1997a, with permission.*

abrupt acceleration of the rate, recorded simultaneously from an epicardial and an M cell. Following acceleration of the rate (change of BCL from 8000 to 800 ms), an abrupt prolongation of APD was observed in the M cell during the initial beats. This was followed by a progressive decrease in the APD, which reached a steady-state only after 55 beats paced at a BCL of 800 ms. In contrast, the epicardial cell displayed only a progressive abbreviation of the APD, which reached a steady-state within 12 beats paced at a shorter BCL. The transient increase in APD following acceleration led to a marked increase in the TDR and the development of EAD activity in the M cells, as illustrated in Figure 4B. The paradoxical d-sotalol-induced action potential prolongation and EAD activity following acceleration is similar to the results obtained with the I_{Kr} blocker E-4031 [51]. The data correlate well with the findings from recent experimental and clinical studies showing that the precipitation of TdP is often preceded by an increase in rate and that acceleration-induced torsade episodes are often accompanied by the appearance of EAD-like deflections in the monophasic action potential [52,53]. Other preliminary studies have postulated an increase in the sodium-calcium exchanger, $I_{Na\text{-}Ca}$, as a basis for acceleration-induced EAD and APD prolongation following I_{Kr} block [54].

While d-sotalol shares its propensity to proarrhythmia with most Class III agents, including the development of TdP, a recent report indicated that the sodium channel blocker mexiletine antagonizes the d-sotalol-induced prolongation of the QT interval and its proarrhythmic effects in an *in vivo* experimental model of TdP [55], suggesting that a combination of low doses of sodium blockade and I_{Kr} block may constitute a useful tool in the pharmacologic treatment of ventricular arrhythmias and help avoid the proarrhythmic effect of Class III agents. This finding is consistent with previous reports which demonstrated that exposure of Purkinje fibres to low concentrations of mexiletine reverses the d-sotalol-induced lengthening of the APD and prevents the EAD activity [56,57].

Although the effectiveness of sodium channel blockers in suppressing TdP has been reported in patients with acquired Long QT Syndrome (LQTS) [58], there have been no reports as regards their effect on TdP in patients with congenital LQTS.

Recent genetic linkage analysis studies have identified at least three forms of congenital LQTS caused by mutations in ion channel genes located on chromosomes 3 (*SCN5A*) (LQT3 syndrome), 7 (*HERG*) (LQT2 syndrome), and 11 (*KvLQT1*) (LQT1 syndrome) [59–61]. The consequences of these three mutations have been shown to result in incomplete inactivation of the sodium channel (LQT3) [59], and in an impaired flow of ions through the potassium channels responsible for I_{Kr} (LQT2) [62] and I_{Ks} (LQT1) [63,64]. Both result in a weaker net outward current and a prolongation of the APD, especially in Purkinje and M cells. The genotypic-phenotypic correlation is an important question to clarify the extent to which these genetic mutations display different phenotypic features, and the relative risk of the development of life-threatening arrhythmias, such as TdP. Shimizu and Antzelevitch [65] used pharmacological agents to mimic two genetic defects: the LQT2 and LQT3 syndromes. These authors used arterially perfused wedges of the canine left ventricle pretreated with d-sotalol or ATX-II as surrogates for LQT2 and LQT3, respectively. The perfused-wedge preparation allowed an assessment of the phenotypic expression of changes in ion channel activity on transmembrane activity and on the ECG. These data indicated that the I_{Kr} blocker d-sotalol produced a much greater prolongation of the APD of M cells than of epicardial and endocardial cells in the perfused-wedge preparation too. As a result, d-sotalol increased the QT interval and the TDR and caused a widening of the T wave. Similarly, ATX-II, an agent that slows the inactivation of the sodium channel, markedly prolonged the QT interval, widened the T wave, and caused a sharp rise in the TDR as a result of a greater prolongation of the APD in the M cells. The greater response of the M cells to ATX-II may be due to a larger late sodium current in this cell type [28]. Schwartz et al. [66] reported that mexiletine significantly shortens the QT interval in LQT3 patients, but not in LQT2 patients. Mexiletine is a class IB antiarrhythmic agent that, like lidocaine, shows rapid dissociation kinetics from the sodium channel. Like many sodium channel blockers, at slow rates and relatively low concentrations, the drug is thought to be able to block the late I_{Na}, with little or no effect on the fast sodium current [67]. The results of Shimizu and Antzelevitch in part agree with the clinical observations of Schwartz et al.; they showed that, while mexiletine was more effective in abbreviating the QT interval in the LQT3 (ATX-II) than in the LQT2 (d-sotalol) model, it reduced TDR and prevented the development of spontaneous and programmed stimulation-induced TdP equally in both LQTS models. Their data suggested that the similar effects of mexiletine in decreasing TDR and suppressing TdP in the LQT2 and LQT3 models were due to the preferential effect of the drug in abbreviating the APD of the M cells, where the late I_{Na} is relatively large, and to a comparatively small effect of shortening the APD of the epicardial and endocardial cells, in which the late I_{Na} is small. Since the M cells are electrotonically well coupled to epicardial and endocardial cells in the perfused wedge, the extent to which the actions of mexiletine are cell-type specific could not be quantified, and the direct effect of the drug in suppressing EAD activity could not be demonstrated. Sicouri et al. [15] provided a further test of the above hypothesis by assessing the effects of sodium channel block with mexiletine in tissues isolated from the epicardial, endocardial and M cell regions of the canine left ventricle under conditions that mimic the LQT2 and LQT3 genetic defects with the proved pharmacologic models of

d-sotalol and ATX-II as surrogates for the congenital syndromes. The d-sotalol model is certainly also representative of the acquired form of LQTS. The principal findings of this study are that mexiletine is effective in reducing TDR and abolishing EAD activity in both the d-sotalol and ATX-II models, and that these effects of the drug are secondary to a differential action of sodium channel block in abbreviating the action potential of epicardial, M and endocardial cells isolated from the canine left ventricle. Figure 5 shows that mexiletine (5 μM) readily reversed the ATX-II induced prolongation of the APD in all three cell types (Panel B), and also the d-sotalol-induced prolongation of APD in the M cells, but not in the epicardium or endocardium (Panel A). Its preferential effect in abbreviating the APD of the M cells is probably due to the presence of a larger late I_{Na}, particularly following exposure to ATX-II. As the late I_{Na} is relatively small in the epicardium and endocardium, either in the absence or in the presence of d-sotalol, it is not surprising that the effect of mexiletine in abbreviating APD is fairly modest in these two types of tissues under these conditions (Fig. 5). The effectiveness of mexiletine in reducing TDR in both models is in large part due to its preferential effect in shortening the APD of M cells, suggesting that the use-dependent block of the sodium channel by mexiletine plays only a minor role, if any, in abbreviating APD and reducing TDR. These data provide support for the hypothesis that the effect of mexiletine in reducing TDR and preventing TdP in the perfused wedge preparation [65] is a result of the differential actions of the drug on the three predominant cell types that comprise the ventricular myocardium.

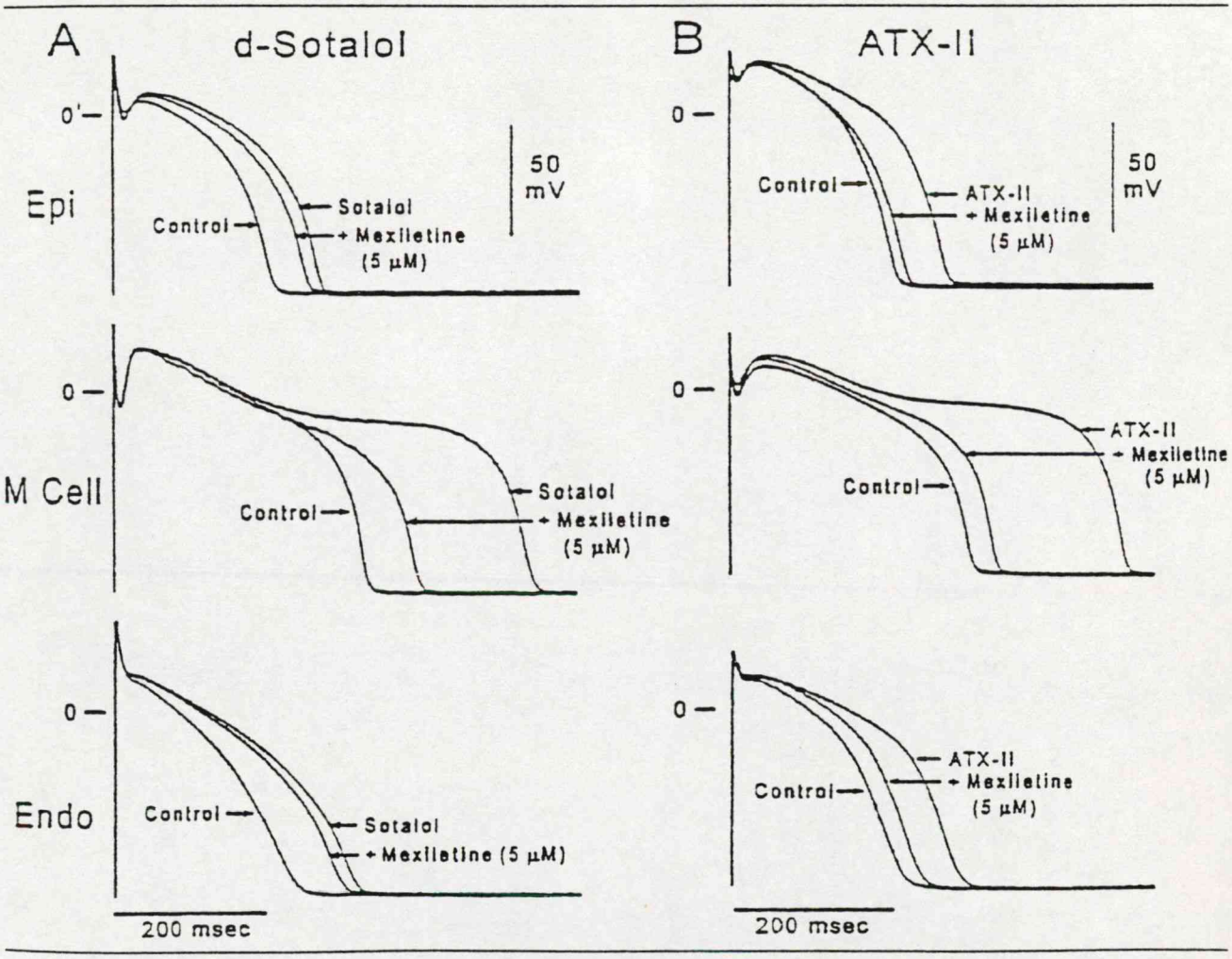

Fig. 5. Effects of mexiletine on action potential characteristics of canine ventricular epicardial (Epi), M cell, and endocardial (Endo) preparations pretreated with d-sotalol (A) or ATX-II (B). Transmembrane activity was recorded simultaneously from Epi, M, and Endo preparations at a basic cycle length of 2000 ms. (A) d-Sotalol (100 μM) produces a preferential prolongation of the M cell action potential. Addition of mexiletine (5 μM) abbreviates the action potential, exerting a much greater effect on the M cell. (B) ATX-II (10 nM) also produces a preferential prolongation of the M cell action potential. Mexiletine (5 μM) abbreviates the APD of the three cell types to near control values. From Sicouri et al. 1997b, with permission.

These data also point to the potential use of agents such as mexiletine in the formulation of an antiarrhythmic cocktail that will produce a uniform prolongation of refractoriness in the ventricular myocardium.

Another frequently used antiarrhythmic drug that causes a reduction in repolarizing K^+ currents and thus prolongs ventricular repolarization and exposes the patients to the risk of QT-dependent arrhythmogenesis is quinidine, that has Class IA properties. TdP has been commonly observed in patients on quinidine who also develop hypokalaemia and present with slow heart rates or long pauses. These conditions are similar to those under which quinidine induces EADs and triggered activity in isolated Purkinje fibres and M cells [13,68]. In this context, it is worthy of note that quinidine blocks I_{Kr} more effectively than the sodium current. This may explain why quinidine often induces TdP at relatively low serum concentration, ie. the torsadogenic effect of this drug does not correlate well with its serum concentration. In contrast, the d-sotalol-induced occurrence of TdP exhibits a reasonable correlation with its plasma level.

A recent addition to the group of pharmacologic agents that can cause the clinical syndrome of acquired long QT, and hence also EADs and TdP, is the widely prescribed macrolide antibiotic erythromycin. Antzelevitch et al. [32] examined the effects of this drug in isolated myocytes, tissues and intact left ventricular wall preparations so as to integrate information at these various levels. Their voltage-clamp results pointed to the erythromycin-induced block of I_{Kr} as a prominent mechanism contributing to the prolongation of repolarization caused by this drug. In isolated strips of tissue, erythromycin (10 to 100 μg/ml) produced a much more pronounced prolongation of the APD in M cells than in endocardial and epicardial cells, resulting in the development of a large dispersion of repolarization across the ventricular wall at slow stimulation rates. The drug (50 to 100 μg/ml) induced EADs in cells of the M region (20%) but not in the epicardial or endocardial regions of the ventricular wall. Erythromycin (100 μg/ml) also caused APD prolongation and a TDR, but not EADs, in intact arterially perfused wedges of canine left ventricle. These changes were accompanied by the development of a long QT interval in the transmural ECG. Polymorphic ventricular tachycardia closely resembling TdP was readily and reproducibly induced after erythromycin, but not before. As EAD activity was observed in only 20% of the M cell tissues and in none of the endocardial or epicardial preparations, it is not suprising that the drug produced no EADs in the arterially perfused left ventricular wedge, where the electrotonic influences of the epicardium and endocardium would be expected to diminish the extent of APD prolongation and prevent the appearance of EADs in the M region.

In other studies, it was reported that erythromycin evoked EADs in the Purkinje fibres [69] and that they were abolished by mexiletine [70]. In this latter paper, a case report included a suspension of TdP after mexiletine administration in a hypokalaemic patient treated with a high dose of erythromycin. These results indicate that erythromycin, like other APD-prolonging agents, targets both the Purkinje fibres and the M cells in the deep structures of the ventricular myocardium.

B) Action potential prolongation and EADs as a result of an increase in the availability of the inward Ca^{2+} current

Sicouri and Antzelevitch [12] used the calcium agonist Bay K 8644 to test the hypothesis that the induction of prominent EADs and triggered activity in the M region of the canine ventricular myocardium can be attained by an increase of the inward Ca^{2-} current. They found that, after a 30-min. exposure to Bay K 8644 (1 μM), the action potential was prolonged in all three myocardial tissue types, but much more so in the M cells than in the epicardium or endocardium. Prominent EADs and EAD-induced triggered activity were demonstrated at the slower stimulation rates (BCL = 1500–5000 ms) in the M cell preparations, but not in the epicardium or endocardium. Unexpectedly, the action potentials recorded from M cells also displayed delayed afterdepolarizations (DADs) after exposure to Bay K 8644. The DADs became more pronounced with deceleration, and multiple DADs were observed at very slow rates. Those authors found that, following acceleration of the stimulation rate to a BCL of 500 ms, the EADs were abolished, but the DADs persisted in the M cells. Further acceleration to a BCL of 300 ms led to more prominent DADs and DAD-induced triggered activity. These results with Bay K 8644 in M cells are in part consistent with those of January et al. [71] in canine and sheep Purkinje fibres. In both studies, the EADs and triggered activity were shown to arise from the plateau of the action potential and to be accentuated with deceleration. The distinction between the effects of Bay K 8644 in Purkinje fibres and M cells is that DADs were easily induced in M cells, but were not observed in Purkinje fibres [71]. The simultaneous occurrence of EADs and DADs over a wide range of frequencies has also been observed in canine myocytes exposed to isoproterenol [72], and in M cells exposed to isoproterenol after pretreatment with E-4031 [73]. In both Purkinje fibres and M cells, Bay K 8644-induced EADs are thought to arise as a consequence of an enhancement of the L-type Ca^{2+} "window" current [74].

A study of the effects of the β-adrenergic agonist isoproterenol on the different cell types pretreated with the I_{Kr} blocker E-4031 [73] revealed that E-4031 significantly prolonged APD in all tissues, but induced EADs in Purkinje fibres (10/13) and M cells (14/17) only at relatively long BCLs. At a BCL of 1000 ms, M cells devoid of EADs displayed a biphasic response to isoproterenol (1 μM): APD was prolonged during the first 1–2 min and then shortened. In contrast, isoproterenol

produced only an abbreviation of the APD in Purkinje, epicardial and endocardial cells. In both Purkinje and M cells, isoproterenol abolished the EADs and DADs at slow rates, but induced both EADs and DADs at fast rates. In M cell preparations, isoproterenol produced alternations of APD at fast rates resulting from EAD and DAD alternations. The drug produced DADs, but not EADs, in epicardial and endocardial cells. These data suggest that β-adrenergic agonists may transiently exaggerate the TDR and promote EAD activity when I_{Kr} is blocked, mimicking the situation in patients with the chromosome 7 genetic defect (HERG), one form of the congenital long QT syndrome (LQT2), or the acquired (Class III antiarrhythmics) long QT syndrome.

Another important pharmacological distinction of M cells and Purkinje fibres is their opposite responses to α-adrenergic agonists [75]. Alpha-agonists, including methoxamine and phenylephrine, produce a prolongation of the Purkinje APD, but an abbreviation of the M cell APD.

In this context, however, it should be mentioned that the increase of the inward calcium current due to isoproterenol or any agent which acts through elevation of the intracellular cAMP level involves a complex effect. Increase of the intracellular cAMP concentration influences other currents, such as the cAMP-dependent chloride current (I_{Cl}) and I_{Ks}, i.e., currents which would shorten the APD. Since the pharmacology of these channels in the different ventricular cell types has not yet been explored, the question of how isoproterenol or sympathetic stimulation influences the dispersion of repolarization or EAD formation can not be answered.

C) An increase in the late Na^+ current, as a source of arrhythmias in M cells

It has already been mentioned that ATX-II, an agent that augments the late I_{Na} by slowing the inactivation of the sodium channel, exerts a preferential effect in M cells and in Purkinje fibres, producing a much more accentuated prolongation of the APD in these cells than in the epicardium or endocardium [15,65]. Anthlopleurin A has a mode of action similar to that of ATX-II, and was used in the recent study of El-Sherif et al. [76] to induce polymorphic ventricular tachycardia with characteristics of TdP in a canine *in vivo* model of LQTS.

TdP is an atypical polymorphic ventricular tachycardia most often associated with prolongation of the QT interval in both congenital and acquired LQTS. The *in vivo* studies of El-Sherif et al. [76] and by Weissenburger et al. [77] involving intramural recordings in the dog, demonstrated the appearance of a marked increase in TDR following exposure to repolarization-prolonging agents and implicated subendocardial Purkinje fibres as the source of focal arrhythmia, and the M cells as the source of the repetitive reentrant excitation circuits observed in torsade episodes following exposure to Anthlopleurin A.

Action potential prolongation and the occurence of TdP has also been reported by the use of ibutilide, an agent that based on data originating from guinea pig myocytes, was also described to increase the late I_{Na} [78]. Others, however, found that the repolarization-prolonging effect of ibutilide—that shares structural similarities with d-sotalol—is rather due to its I_{Kr} blocking property [79,80].

Eddlestone et al. [28] characterized the late or slowly inactivating sodium current in myocytes isolated from the epicardial and M regions of the canine left ventricular free wall and found that the M cells exhibited a significantly larger such sodium current than that of the epicardial cells. Similar results are known for Purkinje fibres [81,82]. These findings can serve as an explanation of why agents that delay the inactivation of the late sodium current target the M cells and Purkinje fibres and produce an exaggerated prolongation of their APD relative to those of the epicardial and endocardial cells. The results of Baláti et al. [24] agree with the data of Eddlestone et al. [28] demonstrating that the late sodium channel inhibitor tetrodotoxin (2 μM) significantly shortened the APD of M cells and Purkinje fibres, but did not appreciably influence this parameter in the epicardial and endocardial cells.

It can be concluded therefore, that a larger late sodium current in M cells is likely to exacerbate the effect of a weaker I_K and thereby contribute to the ability of the M cell action potential to undergo dramatic prolongation at slow rates and in response to agents with Class III actions.

Agents That Diminish Transmural Dispersion of Repolarization

The different responses of the four functionally distinct cell types of the ventricles to all the pharmacological agents mentioned so far in this work lead to a further increase in the electrical heterogeneity of these cardiac chambers, consisting of tissues that are characterized by action potentials considerably different from each other in duration even under control conditions. In other words, TDR is further exaggerated as a result of the effects of a number of clinically widely used agents.

Amiodarone is regarded nowadays as the most effective drug available for cardiac rhythm disturbances, exhibiting a uniquely complex spectrum of electropharmacological actions, the properties of which belong to all four antiarrhythmic classes. It has proved in large clinical trials to be an extremely potent antiarrhythmic agent, suppressing both ventricular and supraventricular arrhythmias. In addition, the results of some studies showed that it was able to reduce significantly mortality due to sudden cardiac death [49]. While most Class III agents have significant

proarrhythmic potency, chronic amiodarone therapy appears to be a rare exception. The recent study by Sicouri and coworkers indicated that chronic amiodarone treatment produced a considerable prolongation of the APD in the epicardium and endocardium of the canine ventricle, but a lesser increase of the APD, or even a decrease at slow rates, in the M cells, thereby reducing TDR (Fig. 6) [16]. Further, chronic amiodarone therapy suppressed the ability of the I_{Kr} blocker d-sotalol, to induce a marked dispersion of repolarization or EAD activity.

These data demonstrated for the first time the direct effect of chronic amiodarone treatment to alter differentially the cellular electrophysiology of the ventricular myocardium so as to produce an important decrease in TDR, especially under conditions in which dispersion is exaggerated. The beneficial APD-shortening effect of the drug is known from previous studies, in which chronic amiodarone treatment did not lengthen, but even abbreviated the repolarization in canine Purkinje fibres, thereby reducing the differences in APD between Purkinje fibres and ventricular muscle (Fig. 7) [83,84]. Further, the EAD induced by I_{Kr} block was abolished by the acute superfusion of 5 μM amiodarone in canine Purkinje fibres (Fig. 8) [84]. It is likely that the APD-shortening effect of amiodarone in both M cells and Purkinje fibres could be due to the depression of the late sodium current [81,85]. Since the plateau sodium current plays a less important role in controlling repolarization in epicardial and endocardial cells than in Purkinje fibres [82] and M cells [28], amiodarone can shorten APD in Purkinje fibres and M cells, whereas in epicardial and endocardial cells the drug lengthens repolarization as a consequence of its inhibitory action on I_{Kr} and perhaps I_{to} [86]. Also, the observed lack of a reverse use dependency in both Purkinje and M cells after amiodarone application might be related to the depression of the late I_{Na}, moderating the prolongation of APD at slower rates. Up to now the effects of amiodarone treatment on M cells in the human heart were not known. The very recent study of Drouin et al. [87] indicated that chronic treatment with amiodarone reduced the TDR also in human heart [87]. These authors found that tissues isolated from the ventricles of heart failure patients receiving chronic amiodarone therapy displayed M cell APD significantly briefer than that recorded in tissues isolated from normal hearts or form heart failure patients not treated with amiodarone. Endocardial cells from amiodarone-treated heart failure patients displayed longer APD than endocardial cells isolated from normal hearts. As a consequence, the heterogeneity of ventricular repolarization in tissues from patients treated with amiodarone was considerably smaller than in the two other groups, especially at long pacing cycle lengths. These results have important clinical implications. It is now recognized that after amiodarone treatment the incidence of TdP arrhythmias is significantly less than after treatment with other Class III antiarrhythmic drugs [88]. It is also well established that all known Class III antiarrhythmics, except amiodarone, increase APD more in Purkinje and M cells than in epicardial or endocardial cells. Furthermore, most Class III drugs, but not amiodarone, exhibit a strong reverse use-dependent effect on repolarization, especially in Purkinje fibres and M cells. If it is assumed that TdP ventricular tachycardia is triggered by EADs originating in Purkinje fibres or M cells [76], it may be speculated that the shortening of repolarization and abolition of EADs by amiodarone in these two particular cell types explains why amiodarone has less potential for the induction of TdP than other Class III antiarrhythmic drugs. The results obtained in the studies by Sicouri et al. [16], Varró et al. [86], Papp et al. [84] and Drouin et al. [87] contribute to our understanding not only of the antiarrhythmic efficacy, but also of the low incidence of proarrhythmia

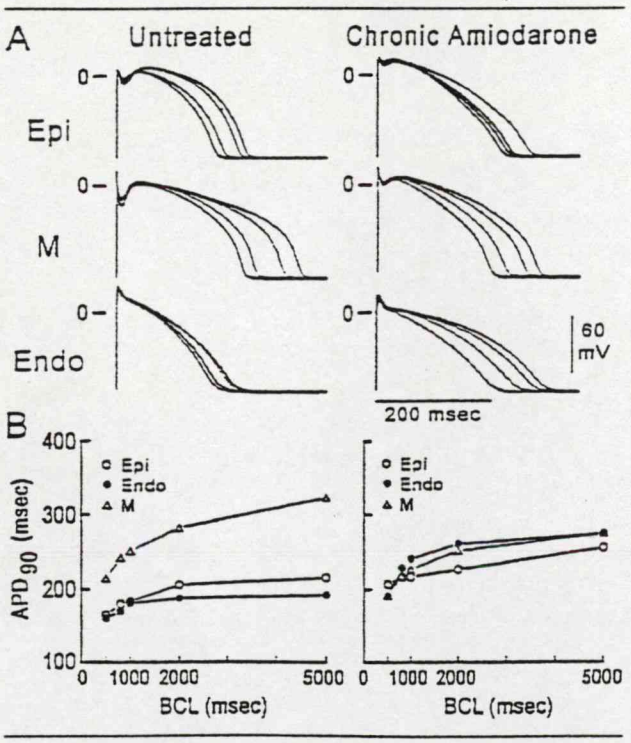

Fig. 6. Effects of chronic amiodarone on the rate dependence of action potential characteristics in epicardial (Epi), M, and endocardial (Endo) preparations isolated from the hearts of untreated dogs (left) and of those receiving chronic amiodarone therapy (right). (A) Transmembrane activity recorded simultaneously from Epi, M, and Endo preparations from the left ventricle of untreated (left) and chronic amiodarone-treated dogs (right). Recordings were obtained at basic cycle lengths (BCLs) of 500, 800, 2000 and 5000 ms under steady-state conditions. (B) Graphic display of action potential duration measured at 90% repolarization (APD_{90}) - rate relations at each site in the same experiment. In amiodarone-treated preparations, action potentials are more prolonged in Epi and Endo, and the rate dependence of APD is less pronounced in the M cell. $[K^+]_o = 4$ mM. From Sicouri et al. 1997c, with permission.

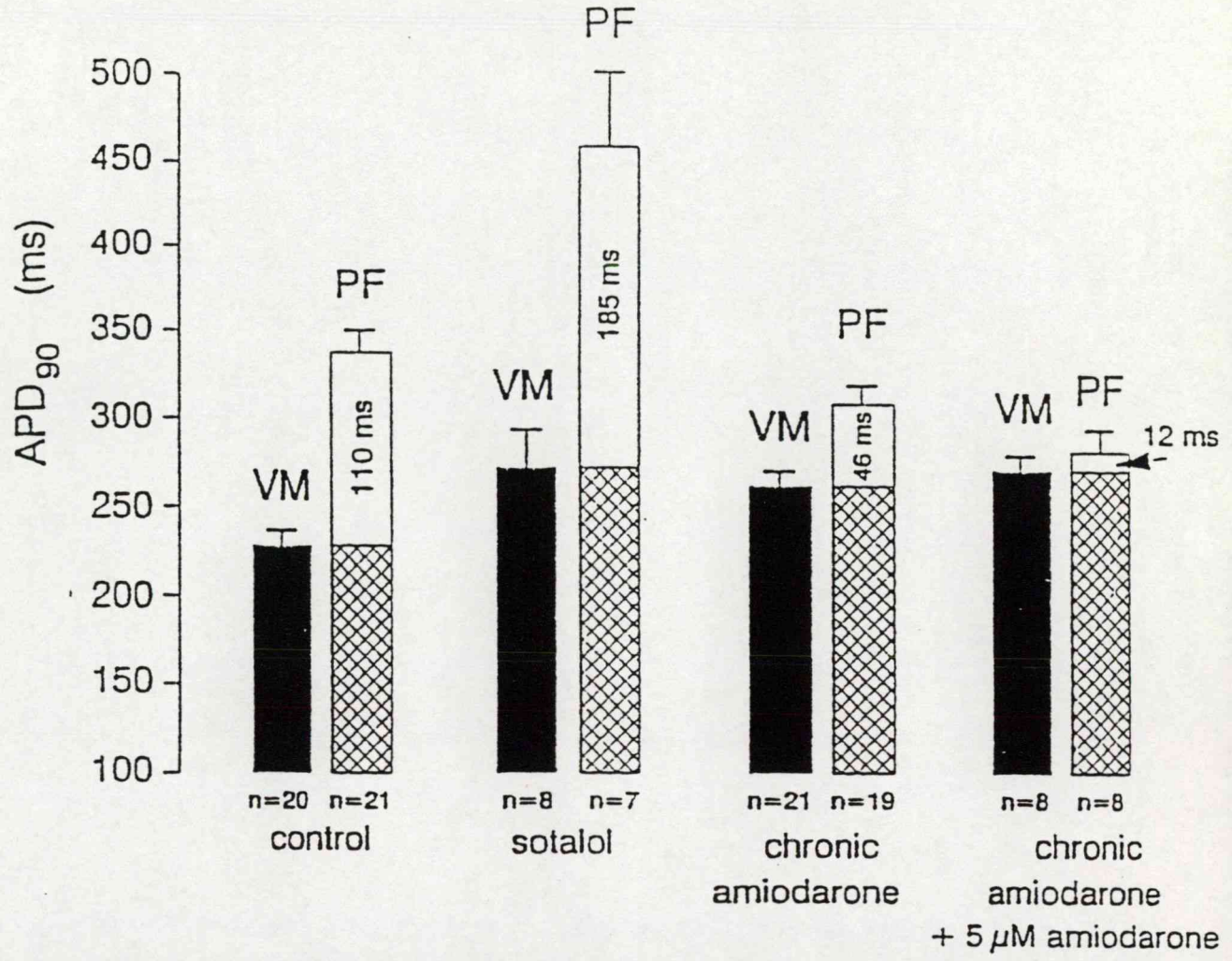

Fig. 7. *The action potential duration differences between Purkinje fibres (PF) and ventricular muscle (VM) during control conditions, in the presence of 30 μM d-sotalol and after chronic amiodarone treatment in the absence or in the presence of amiodarone. The stimulation frequency was 1 Hz, and bars represent ± standard errors. From Papp et al. 1996, with permission.*

accompanying therapy with chronic amiodarone in comparison with other Class III agents.

Another agent recently shown to be effective in diminishing TDR is sodium pentobarbital, a drug widely used for anaesthesia. It is of interest that the use of sodium pentobarbital as an anaesthetic resulted in failure in an attempt to develop TdP in *in vivo* models of the long QT syndrome [89,90]. In the recent *in vivo* comparison by Weissenburger et al. [89], the dispersion of repolarization measured across the anterior left ventricular wall (transmural monophasic action potential recordings) was considerably smaller both before and after d-sotalol when sodium pentobarbital was used for anesthesia as compared to halothane. d-Sotalol produced a dramatic increase in TDR when halothane was used for anaesthesia, but not when sodium pentobarbital was employed. TdP occurred only during halothane anaesthesia. Sun et al. [91] demonstrated that an abbreviation of the M cell action potential, but prolongation of the epicardial and endocardial APDs, underlie the effect of sodium pentobarbital in reducing TDR in a control setting and under conditions of an acquired long QT. Those authors also identified the currents sensitive to sodium pentobarbital, in myocytes dissociated from the canine left ventricle. It was found that sodium pentobarbital, at physiologically relevant concentrations, diminished the late I_{Na} in the M cells by approximately 50%, and also decreased I_{Ks} by 50%, I_{Kr} by approximately 20%, and the inward rectifier potassium current (I_{K1}) by 40% in the endocardial cells. These effects of pentobarbital may underlie its differential action on cells spanning the ventricular wall.

It has been reported that in an *in vivo* rabbit TdP model the ATP-sensitive K-channel opener pinacidil

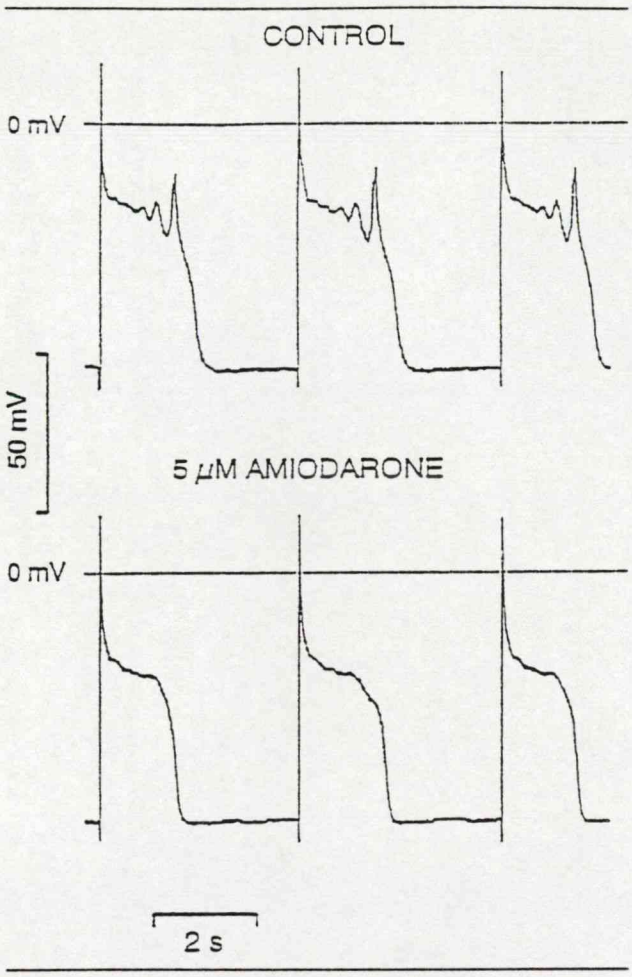

Fig. 8. The effects of 5 μM amiodarone on early afterdepolarizations induced by 1 μM dofetilide, 20 μM $BaCl_2$ and 1 mM CsCl in canine Purkinje fibres. The stimulation frequency was 0.15 Hz. From Papp et al. 1996, with permission.

abolished TdP arrhythmias [92]. Consistent with this finding, it was shown earlier that nicorandil, another ATP-sensitive K-channel activator, eliminated EADs evoked in canine Purkinje fibres [93]. Further, recent data from our laboratory indicated that 10 μM pinacidil shortened APD more in Purkinje fibres and M cells than in endocardial or epicardial preparations [24]. Antzelevitch et al. [94] suggested that nicorandil is capable of abbreviating long QT, reducing TDR and preventing spontaneous and stimulation-induced TdP when LQTS is secondary to a reduced I_{Ks} (LQT1) or I_{Kr} (LQT2), but not when it is due to an augmented late I_{Na} (LQT3), although relatively high concentrations of nicorandil are required [94].

The similar effects of mexiletine in reducing TDR and suppressing TdP in the different models of LQTS have already been discussed above.

It was very recently reported that R-L3, a new investigational benzodiazepine, enhanced I_{Ks} in guinea pig ventricular myocytes [95]. Since the activation of I_{Ks} is relatively slow, it probably plays an important role if APD is lengthened. The densitiy of I_{Ks} was reported to be less in M than in epicardial or endocardial cells [27]. It is tempting to speculate therefore, that the enhancement of I_{Ks} by R-L3 may limit the excessive APD prolongation caused by Class III drugs, particularly in M cells at slow heart rates, causing a considerable reduction of the enhanced dispersion of repolarization.

Conclusions

The discovery of M cells in the ventricular wall of most mammalian species has prompted a reevaluation of some existing concepts concerning the electrophysiology, pharmacology and pathophysiology of the cardiac ventricular muscle. A significant consequence of the prominent electrophysiological heterogeneity existing among the four types of tissue in the ventricles is that they respond differently to a wide variety of pharmacological interventions. Therefore we compared the pharmacological response of the four tissue types to 3 main groups of agents that act to enhance TDR in the ventricles, and to agents that have the beneficial effect of decreasing it. In the past, our interest was focused on how arrhythmias could be suppressed effectively by drugs which lengthen repolarization. M cells differ primarily from the other two ventricular muscle cell types, but resemble Purkinje fibres, in their ability to prolong the APD dramatically in response to agents that lengthen repolarization and thus enhance further TDR that exists in the ventricles also even under physiological conditions. In recent years, the focus of our interest shifted from effectivity in lengthening repolarization towards the safe achievement of this. At present amiodarone seems to be the only currently used antiarrhythmic drug that has the ability to produce a significant decrease in TDR at all BCLs, by prolonging repolarization in endocardial and epicardial cells, lacking to influence it considerably in M cells and shortening it in Purkinje fibres. The characterization of M cells and a better understanding of the dispersion of repolarization may promote the future development of new antiarrhythmic strategies. The combination of I_{Kr} blockers with sodium channel inhibitors that do not affect conduction at a normal heart rate (Class I/B type) seems to be a possible approach. Further, the combination of cardioselective ATP-sensitive K-channel openers with Class III drugs may afford a therapeutic advantage. The recently developed I_{Ks} activator drug combined with I_{Kr} blockers may offer a unique opportunity for the induction of repolarization lengthening at normal and fast rates without increasing TDR and thereby imposing less proarrhythmic risk. 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.

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VI

The role of the delayed rectifier component I_{Ks} in dog ventricular muscle and Purkinje fibre repolarization

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1. The relative contributions of the rapid and slow components of the delayed rectifier potassium current (I_{Kr} and I_{Ks}, respectively) to dog cardiac action potential configuration were compared in ventricular myocytes and in multicellular right ventricular papillary muscle and Purkinje fibre preparations. Whole-cell patch-clamp techniques, conventional microelectrode and *in vivo* ECG measurements were made at 37 °C.

2. Action potential duration (APD) was minimally increased (less than 7%) by chromanol 293B (10 μM) and L-735,821 (100 nM), selective blockers of I_{Ks}, over a range of pacing cycle lengths (300–5000 ms) in both dog right ventricular papillary muscles and Purkinje fibre strands. D-Sotalol (30 μM) and E-4031 (1 μM), selective blockers of I_{Kr}, in the same preparations markedly (20–80%) lengthened APD in a reverse frequency-dependent manner.

3. *In vivo* ECG recordings in intact anaesthetized dogs indicated no significant chromanol 293B (1 mg kg^{-1} I.V.) effect on the QTc interval (332·9 ± 16·1 ms before *versus* 330·5 ± 11·2 ms, $n = 6$, after chromanol 293B), while D-sotalol (1 mg kg^{-1} I.V.) significantly increased the QTc interval (323·9 ± 7·3 ms before *versus* 346·5 ± 6·4 ms, $n = 5$, after D-sotalol, $P < 0·05$).

4. The current density estimated during the normal ventricular muscle action potential (i.e. after a 200 ms square pulse to +30 mV or during a 250 ms long 'action potential-like' test pulse) indicates that substantially more current is conducted through I_{Kr} channels than through I_{Ks} channels. However, if the duration of the square test pulse or the 'action potential-like' test pulse was lengthened to 500 ms the relative contribution of I_{Ks} significantly increased.

5. When APD was pharmacologically prolonged in papillary muscle (1 μM E-4031 and 1 μg ml^{-1} veratrine), 100 nM L-735,821 and 10 μM chromanol 293B lengthened repolarization substantially by 14·4 ± 3·4 and 18·0 ± 3·4% ($n = 8$), respectively.

6. We conclude that in this study I_{Ks} plays little role in normal dog ventricular muscle and Purkinje fibre action potential repolarization and that I_{Kr} is the major source of outward current responsible for initiation of final action potential repolarization. Thus, when APD is abnormally increased, the role of I_{Ks} in final repolarization increases to provide an important safety mechanism that reduces arrhythmia risk.

The delayed rectifier potassium current (I_K) is a major outward current responsible for ventricular muscle action potential repolarization (Carmeliet, 1993; Sanguinetti & Keating, 1997). This current was first described by Noble & Tsien (1969) using the two-microelectrode voltage-clamp technique in multicellular sheep cardiac Purkinje fibre strands. Since its discovery it has been examined in single isolated myocytes obtained from various regions of the heart in several mammalian species (Noble & Tsien, 1969; Sanguinetti & Jurkiewicz, 1990; Follmer & Colatsky, 1990; Varró *et al.* 1993; Gintant, 1996; Salata *et al.* 1996*a*). In most species, I_K can be separated into rapid and slow

components (I_{Kr} and I_{Ks}, respectively) that differ from one another in terms of their sensitivity to drugs, rectification characteristics, and kinetic properties (Sanguinetti & Jurkiewicz, 1990; Carmeliet, 1992; D. W. Liu & Antzelevitch, 1995; Gintant, 1996; Heath & Terrar, 1996*a*,*b*). Specific I_{Kr} blockers (e.g. D-sotalol, dofetilide and E-4031) greatly lengthen cardiac action potential duration (APD) (Singh & Vaughan Williams, 1970; Strauss *et al.* 1970; Lathrop, 1985; Jurkiewicz & Sanguinetti, 1993) and thus provide anti-arrhythmic benefit by increasing the refractory wavelength. However, these drugs also increase risk for development of bradycardia-induced polymorphic ventricular tachy-arrhythmias (Hohnloser & Woosley, 1994). The APD increase induced by selective I_{Kr} blockade displays 'reverse use dependency' (Hondeghem & Snyders, 1990) that is especially pronounced in Purkinje fibres (Varró *et al.* 1985). Thus, with a premature impulse, when the time between successive depolarizations is short and an increase in APD would provide the most anti-arrhythmic benefit, the actual APD increase due to I_{Kr} block is the least. Conversely, when the time between successive action potentials is long, as with slow heart rates, selective I_{Kr} block produces a far greater increase in APD. Long APDs such as these are often associated with the development of early after-depolarizations that are probably responsible for induction of Torsade de Pointes ventricular arrhythmias.

The absence of selective I_{Ks} blockers until recently has made it impossible to evaluate directly the physiological role of this current in determining action potential configuration. Nevertheless, selective I_{Ks} block has generally been assumed to increase APD and refractoriness in a frequency-independent manner. On this basis, there has been an effort to develop selective I_{Ks} blockers as potential anti-arrhythmic agents devoid of the risk of Torsade de Pointes arrhythmia induction. Propofol, thiopenthone (Heath & Terrar, 1996*a*) and indapamide (Turgeon *et al.* 1994) were first used as pharmacological tools to block I_{Ks} and thereby separate I_{Ks} from I_{Kr} in guinea-pig ventricular myocytes. These compounds, however, effectively block I_{Ks} at concentrations higher than 100 μM, which calls into question their I_{Ks} selectivity. Two compounds, chromanol 293B (Busch *et al.* 1996) and L-735,821 (Salata *et al.* 1996*b*; Cordeiro *et al.* 1998) have recently been reported to selectively block I_{Ks}, but their effects on cardiac action potential configuration have not been examined in detail. Moreover, available results obtained with chromanol 293B and with L-735,821 are contradictory. Cordeiro *et al.* (1998), for instance, found L-735,821 to markedly increase APD in single, isolated rabbit Purkinje fibre myocytes. Bosch *et al.* (1998) similarly found chromanol 293B to increase APD in guinea-pig and human ventricular myocytes. However, conventional microelectrode recordings in guinea-pig right papillary muscle showed that chromanol 293B only slightly lengthened APD in the absence of isoproterenol (isoprenaline) (Schreieck *et al.* 1997). These contradictory findings may have several explanations. For one, APD measurements in single myocytes inherently show relatively large beat-to-beat variations that make identification of the effects of selective ion channel block on action potential configuration uncertain at best. In addition, the relative expression of I_{Kr} and I_{Ks} exhibits considerable species variation (Jurkiewicz & Sanguinetti, 1993; Varró *et al.* 1993; Li *et al.* 1996; Salata *et al.* 1996*a*). Regional differences in ion channel expression within the ventricle (Antzelevitch *et al.* 1991; Bryant *et al.* 1998) probably also confound interpretation of results and lead to differences in the effects of selective ion channel blockade in myocytes isolated from whole hearts.

Because I_{Ks} activation occurs at around 0 mV and this voltage is more positive than the normal Purkinje fibre action potential plateau voltage, I_{Ks} block should not be expected to increase Purkinje fibre APD. Conversely, in ventricular muscle, action potential plateau voltage is more positive (~ +20 mV) allowing I_{Ks} to be substantially more activated. Thus I_{Ks} block in ventricular muscle would be expected to increase APD markedly. Such a difference in the effects of I_{Ks} block might, therefore, be expected to produce anti-arrhythmic benefit. This is because lengthening ventricular muscle APD with little or no change in Purkinje fibre APD would cause less drug-induced dispersion in repolarization and limit arrhythmogenesis.

The main goal of this study was to compare the magnitude and extent of changes in ventricular muscle APD produced by selective block of I_{Kr} and I_{Ks} with those effects produced in Purkinje fibres. The results from such studies would establish the role of I_{Ks} in producing normal cardiac action potential repolarization. Thus, we compared the effects of two purported I_{Ks} blockers (chromanol 293B and L-735,821) with the effects produced by two recognized, selective I_{Kr} blockers (E-4031and D-sotalol) in both single myocytes and multicellular cardiac preparations.

METHODS

All experiments were approved by the Hungarian National Research Foundation (OTKA) and conducted in compliance with the Guide for the Care and Use of Laboratory Animals (USA NIH publication No. 85-23, revised 1985).

Conventional microelectrode measurements

Adult mongrel dogs of either sex weighing 8–16 kg were used. Following anaesthesia induced by sodium pentobarbital (30 mg kg^{-1} I.V.), each heart was rapidly removed through a right lateral thoracotomy and immediately rinsed in oxygenated modified Locke's solution containing (mM): Na^+ 140, K^+ 4, Ca^{2+} 1·0, Mg^{2+} 1, Cl^- 126, HCO_3^- 25 and glucose 11. The solution pH ranged from 7·35 to 7·45 when gassed with 95% O_2–5% CO_2 at 37 °C. Purkinje strands obtained from either ventricle and right ventricular papillary muscle tips were mounted individually in a tissue chamber (volume ~40 ml). Each preparation was stimulated (HSE stimulator type 215/II) initially at a constant cycle length of 1000 ms (frequency 1 Hz) using rectangular constant current pulses of 2 ms in duration. The current pulses were isolated from ground

and delivered through bipolar platinum electrodes in contact with the preparations. At least 1 h was allowed for each preparation to equilibrate while continuously superfused with modified Locke's solution warmed to 37 °C before experimental measurements commenced. Transmembrane potentials were recorded using conventional 5–20 MΩ, 3 M KCl-filled microelectrodes connected to the input of a high impedance electrometer (Biologic Amplifier VF 102, Claix, France). In addition, the first derivative of transmembrane voltage with respect to time (V_{max}) was electronically obtained (Biologic Differentiator DV 140, Claix, France) and, along with the transmembrane voltage amplifier outputs, continuously monitored on a dual beam storage oscilloscope (Tektronix model 2230).

The maximum diastolic potential, action potential amplitude and action potential durations at 50% and 90% of repolarization (APD_{50} and APD_{90}) were automatically measured using software developed in our laboratory (Hugo Sachs Elektronik, March-Hugstetten, Germany; action potential evaluation system) running on a 386 microprocessor based, IBM compatible computer, containing an ADA 3300 analog-to-digital data acquisition board (Real Time Devices Inc., PA, USA) with a maximum sampling frequency of 40 kHz. In each experiment, baseline action potential characteristics were first determined during continuous pacing at 1 Hz, and then when pacing cycle length was sequentially varied from 300–5000 ms. The 25th action potential was measured at each cycle length, and the cycle length was then changed so that 'quasi' steady-state frequency response relations could be generated rapidly. Preparations were then superfused for 40–60 min with either drug before repeating the pacing protocol and measuring the same parameters. Attempts were made to maintain the same impalement throughout each experiment. If an impalement was, however, dislodged, electrode adjustment was attempted, and if the action potential characteristics of the re-established impalement deviated by less than 5% from the previous measurement, the experiment continued. When this 5% limit was exceeded, the experiment was terminated and all data were excluded from analyses.

Patch-clamp measurements

Cell isolation. Ventricular myocytes were enzymatically dissociated from hearts which were removed from mongrel dogs of either sex weighing 10–20 kg following anaesthesia (sodium pentobarbital, 30 mg kg^{-1} I.V.). The hearts were immediately placed in cold (4 °C) normal Tyrode solution. A portion of the left ventricular wall containing an arterial branch large enough to cannulate was then perfused in a modified Langendorff apparatus at a pressure of 60 cm H_2O with solutions in the following sequence: (1) normal Tyrode solution (10 min), (2) Ca^{2+}-free solution (10 min), and (3) Ca^{2+}-free solution containing collagenase (type I, 0·66 mg ml^{-1}, Sigma) and bovine serum albumin (fraction V, fatty acid free, 2 mg ml^{-1}, Sigma) (15 min). Protease (type XIV, 0·12 mg ml^{-1}, Sigma) was added to the final perfusate and another 15–30 min of digestion was allowed. Portions of the left ventricular wall judged to be well digested were diced into small pieces and placed either in Kraft-Brühe (KB) solution or in Ca^{2+}-free solution supplemented with $CaCl_2$ (1·25 mM) for 15 min. Next, these tissue samples were gently agitated in a small beaker to dislodge single myocytes from the extracellular matrix. All cell suspensions resulting from this dissociation procedure contained a mixture of subepicardial, midmyocardial and subendocardial myocytes. During the entire isolation procedure, solutions were gassed with 100% O_2 while their temperatures were maintained at 37 °C. Myocytes were allowed to settle to the bottom of the beaker for 10 min, and then half of the supernatant was replaced with fresh solution. This procedure was repeated three times. Myocytes placed in KB solution were stored at 4 °C; those placed in Tyrode solution were maintained at 12–14 °C prior to experimentation. Cells that were stored in KB solution or immediately placed in 1·25 mM calcium containing solution had the same appearance and there were no discernible differences in their characteristics.

Compositions of solutions used for cell isolation. Normal Tyrode solution (mM): NaCl 135, KCl 4·7, KH_2PO_4 1·2, $MgSO_4$ 1·2, Hepes 10, $NaHCO_3$ 4·4, glucose 10 and $CaCl_2$ 1·0 (pH 7·2 adjusted with NaOH). Ca^{2+}-free solution (mM): NaCl 135, KCl 4·7, KH_2PO_4 1·2, $MgSO_4$ 1·2, Hepes 10, $NaHCO_3$ 4·4, glucose 10 and taurine 20 (pH 7·2 adjusted with NaOH). KB solution (mM): KOH 90, L-glutamic acid 70, taurine 15, KCl 30, KH_2PO_4 10, $MgCl_2$ 0·5, Hepes 10, glucose 11 and EGTA 0·5 (pH 7·3 adjusted with KOH).

Experimental procedure, drugs and solutions. One drop of cell suspension was placed within a transparent recording chamber mounted on the stage of an inverted microscope (TMS, Nikon, Tokyo, Japan), and individual myocytes were allowed to settle and adhere to the chamber bottom for at least 5 min before superfusion was initiated. Only rod-shaped cells with clear striations were used. Cell capacitance (199·3 ± 13·7 pF, $n = 69$) was measured by applying 10 mV hyperpolarizing pulse from a holding potential of −10 mV. The capacity was measured by integration of the capacitive transient divided by the amplitude of the voltage step (10 mV). Hepes-buffered Tyrode solution served as the normal superfusate. This solution contained (mM): NaCl 144, NaH_2PO_4 0·33, KCl 4·0, $CaCl_2$ 1·8, $MgCl_2$ 0·53, glucose 5·5 and Hepes 5·0 at pH 7·4.

E-4031 (Institute for Drug Research, Budapest, Hungary) and D-sotalol (Bristol-Arzneimittel, Troisdorf, Germany) were diluted from a 1 mM or 10 mM aqueous stock solution, respectively, at the time of the experiment. Chromanol 293B (obtained as a gift from Hoechst AG, Frankfurt, Germany) was similarly diluted at the time of use from a 10 mM stock solution containing 100% DMSO. DMSO at this concentration did not produce discernible effects either on APD or measured currents. L-735,821 (obtained as a gift from Merck-Sharpe & Dohme Laboratories, Rathway, NJ, USA) was diluted in superfusate from a 100 μM stock solution containing 10% DMSO. Patch-clamp micropipettes were fabricated from borosilicate glass capillaries (Clark, Reading, UK) using a P-97 Flaming-Brown micropipette puller (Sutter Co., Novato, CA, USA). These electrodes had resistances between 1·5 and 2·5 MΩ when filled with pipette solution containing (mM): potassium aspartate 100, KCl 45, K_2ATP 3, $MgCl_2$ 1, EGTA 10 and Hepes 5. The pH of this solution was adjusted to 7·2 with KOH. Nisoldipine (1 μM) (obtained as a gift from Bayer AG, Leverkusen, Germany) was placed in the external solution to eliminate inward Ca^{2+} current (I_{Ca}), and sodium current (I_{Na}) was inactivated by applying a holding potential of −40 mV which also largely inactivated transient outward current (I_{to}). Membrane currents were recorded with an Axopatch-1D amplifier (Axon Instruments, Foster City, CA, USA) using the whole-cell configuration of the patch-clamp technique. After establishing a high (1–10 GΩ) resistance seal by gentle suction, the cell membrane beneath the tip of the electrode was disrupted by suction or by application of 1·5 V electrical pulses for 1–5 ms. The series resistance was typically 4–8 MΩ before compensation (50–80%, depending on the voltage protocols). Experiments where the series resistance was high, or substantially increased during measurement, were discarded. Membrane currents were digitized using a 333 kHz analog-to-digital converter

(Digidata 1200, Axon Instruments) under software control (pCLAMP 6.0, Axon Instruments). Analyses were performed using pCLAMP 6.0 software after low-pass filtering at 1 kHz. All patch-clamp data were collected at 37 °C.

ECG measurements in intact anaesthetized dogs

Adult mongrel dogs of either sex weighing 8–16 kg were anaesthetized using sodium pentobarbital (30 mg kg^{-1} I.V.) with subsequent bolus I.V. injections (6 mg kg^{-1}) administered as needed. These dogs were ventilated with room air at a rate and tidal volume sufficient to maintain arterial O_2, CO_2 and pH within normal limits (Végh *et al.* 1992). Catheters were inserted into the right and left femoral veins for drug and anaesthetic administration. The dose of each drug applied was 1 mg kg^{-1}. Drugs were administered slowly (over a period of 1 min) in a volume equivalent to 0·5 ml kg^{-1}. Surface electrocardiographic (ECG) leads I, II and III were continuously monitored and recorded after 1, 3 and 5 min and every subsequent 5 min during drug administration for up to 30 min. After completion of the experiments, animals were killed by I.V. overdose of pentobarbital.

Statistical analyses

Results were compared using Student's t tests for paired and unpaired data. When $P < 0·05$, results were considered significant. Data are expressed as means ± S.E.M.

RESULTS

Effects of E-4031 and D-sotalol on I_{Kr}

The effects of E-4031 and D-sotalol on I_{Kr} were examined in isolated dog ventricular myocytes. Test pulses of 1000 ms in duration to between −20 mV and +50 mV were applied from a holding potential of −40 mV. The decaying tail current at −40 mV after the test pulse was assessed as I_{Kr}. L-735,821 (100 nM) or chromanol 293B (30 μM) were used to block I_{Ks} completely. Under these conditions, E-4031 (1 μM) completely abolished and D-sotalol (30 μM) attenuated (not shown) I_{Kr} tail currents (Fig. 1).

Effects of chromanol 293B and L-735,821 on I_{Ks}

The effects of chromanol 293B and L-735,821 on I_{Ks} were examined using long (5000 ms) test pulses to between 0 mV and +50 mV from a holding potential of −40 mV in the presence of 1–5 μM E-4031 to inhibit I_{Kr}. The decaying tail current at −40 mV following each test pulse was assessed as I_{Ks}. Chromanol 293B (10 μM) greatly reduced and L-735,821 (100 nM) completely abolished I_{Ks} (Figs 2 and 3).

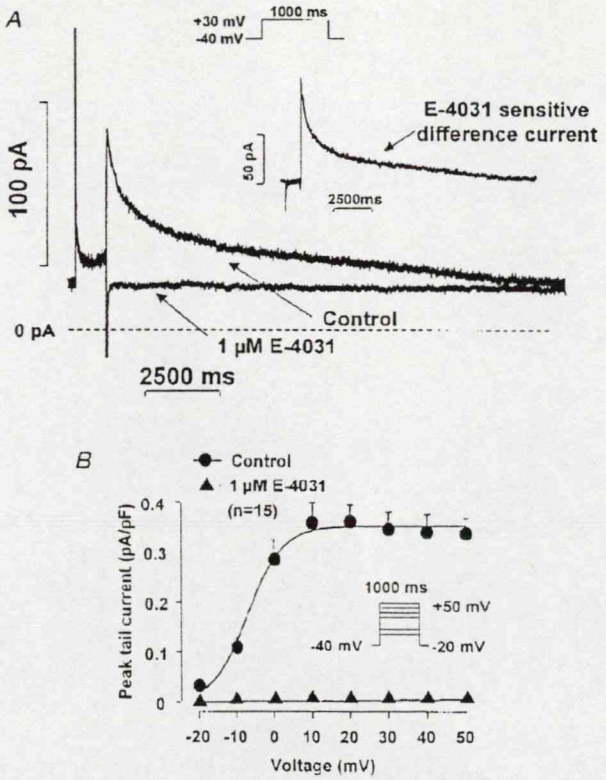

Figure 1. E-4031-sensitive current (I_{Kr}) in dog ventricular myocytes

A, recording of I_{Kr} in the absence and presence of 1 μM E-4031. The inset presents the E-4031 (1 μM)-sensitive difference current at +30 mV. *B*, the peak I_{Kr} tail current amplitude–voltage relationship in the absence and presence of 1 μM E-4031. Nisoldipine (1 μM) was used to block inward I_{Ca} and L-735,821 (100 nM) to block I_{Ks}. Holding potential (V_h) was −40 mV, pulse duration was 1000 ms, and pulse frequency was 0·05 Hz.

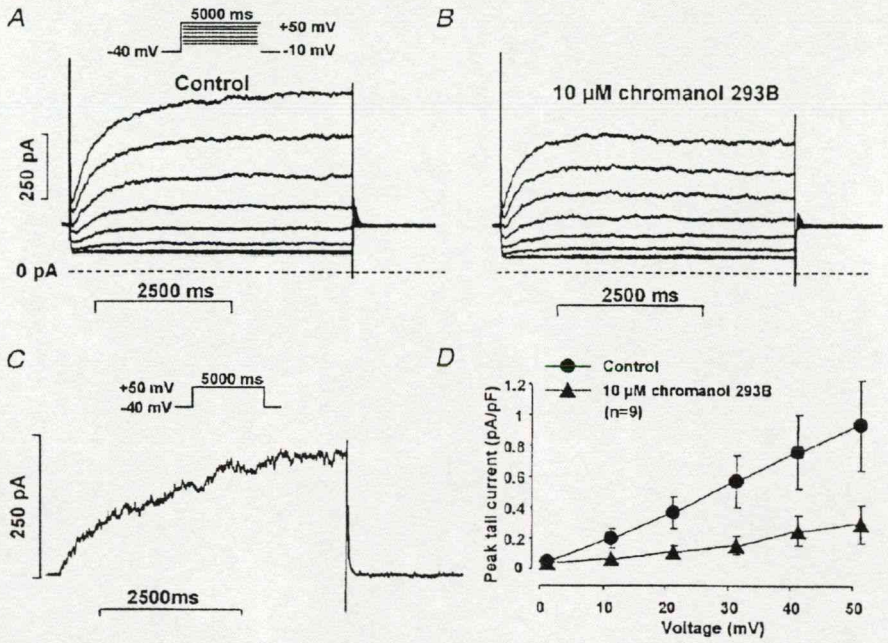

Figure 2. E-4031-insensitive current (I_{Ks}) in dog ventricular myocytes: effect of chromanol 293B
A and *B*, recordings in the absence and presence, respectively, of 10 μM chromanol 293B. *C*, the chromanol 293B (10 μM)-sensitive difference current at +50 mV. *D*, peak I_{Ks} tail current amplitude–voltage relationship in the absence and presence of 10 μM chromanol 293B. Nisoldipine (1 μM) was used to block inward I_{Ca} and E-4031 (5 μM) to block I_{Kr}. V_h was −40 mV, pulse duration was 5000 ms, and pulse frequency was 0·1 Hz.

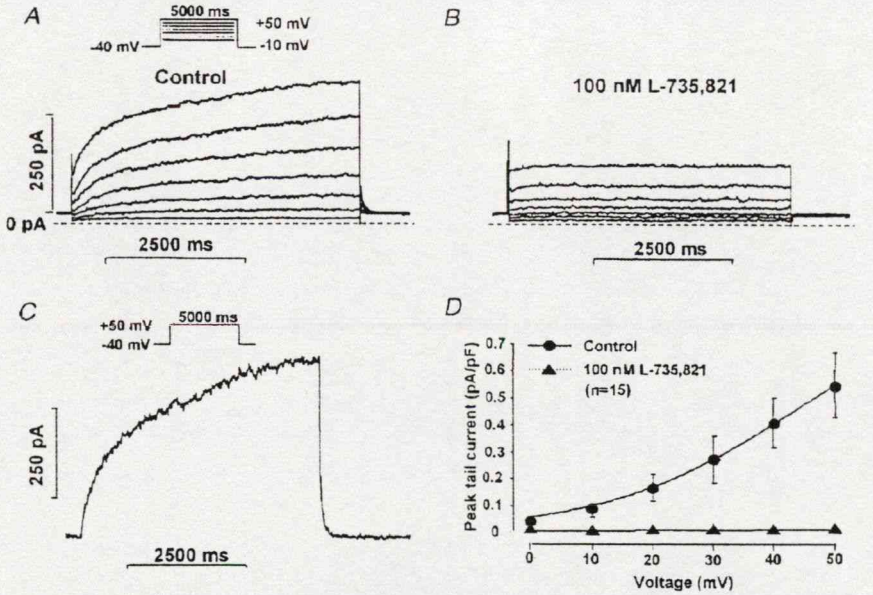

Figure 3. E-4031-insensitive current (I_{Ks}) in dog ventricular myocytes: effect of L-735,821
A and *B*, recordings in the absence and presence, respectively, of 100 nM L-735,821. *C*, the L-735,821 (100 nM)-sensitive difference current at +50 mV. *D*, peak I_{Ks} tail current amplitude–voltage relationship in the absence and presence of 100 nM L-735,821. Nisoldipine (1 μM) was used to block inward I_{Ca} and E-4031 (5 μM) to block I_{Kr}. V_h was −40 mV, pulse duration was 5000 ms, and pulse frequency was 0·1 Hz.

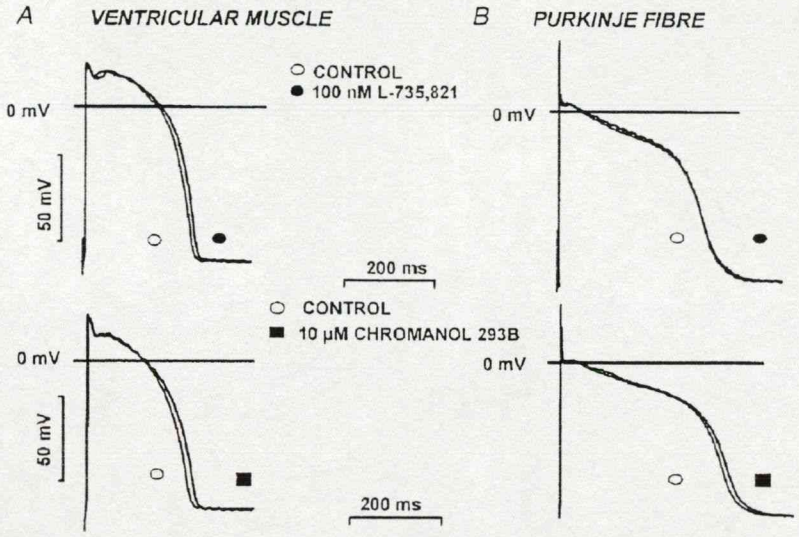

Figure 4. Effect of I_{Ks} block on the action potential in dog ventricular right papillary muscle and Purkinje fibre

Action potential recordings from canine ventricular papillary muscles (*A*) and Purkinje fibre strands (*B*) before and after 40 min superfusion with 100 nM L-735,821 (top) or 10 μM chromanol 293B (bottom). Stimulation frequency was 1 Hz.

Possible contribution of I_{Ks} and I_{Kr} to action potential repolarization in dog ventricular muscle and Purkinje fibres

The effects on dog ventricular muscle and Purkinje fibre action potential configuration produced by equipotent concentrations of chromanol 293B (10 μM) and L-735,821 (100 nM) that blocked I_{Ks} (Fig. 4) were examined and compared with those of D-sotalol (30 μM) and E-4031 (1 μM) that blocked I_{Kr} (Fig. 5). Conventional microelectrode techniques were used and the effects of these compounds that completely or markedly blocked either I_{Ks} or I_{Kr} were examined in both dog ventricular muscle and Purkinje fibre strands over a wide range of stimulation cycle lengths (300–5000 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 fibre strands (Fig. 6). In

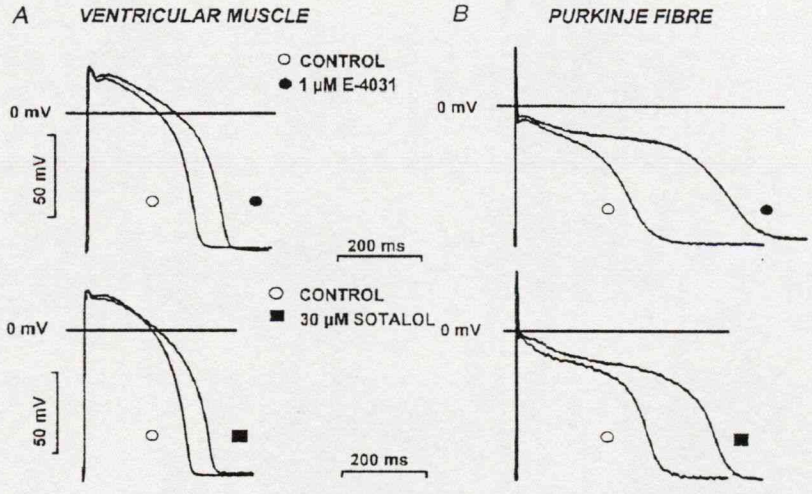

Figure 5. Effect of I_{Kr} block on the action potential in dog ventricular right papillary muscle and Purkinje fibre

Action potential recordings from canine ventricular papillary muscles (*A*) and Purkinje fibre strands (*B*) before and after 40 min superfusion with 1 μM E-4031 (top) or 30 μM D-sotalol (bottom). Stimulation frequency was 1 Hz.

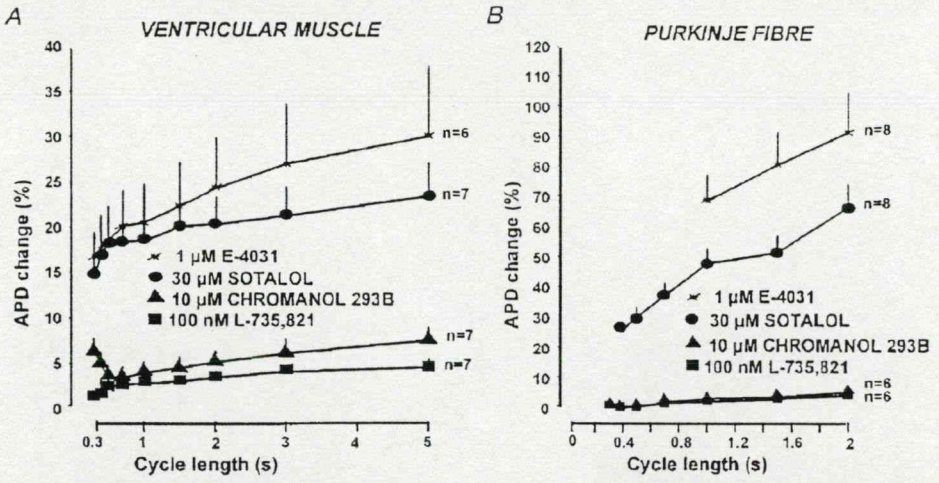

Figure 6. Frequency-dependent effect of I_{Kr} and I_{Ks} block on action potential duration
Frequency-dependent effect of I_{Kr} (by 1 μM E-4031 or 30 μM sotalol) and I_{Ks} block (by 10 μM chromanol 293B or 100 nM L-735,821) on action potential duration (APD) in canine ventricular papillary muscles (*A*) and Purkinje fibre strands (*B*). Pacing cycle length (1/frequency) is plotted on the abscissa and the ordinate indicates percentile changes in APD_{90}. Bars represent S.E.M.

contrast, D-sotalol and E-4031 markedly lengthened both dog papillary muscle and Purkinje fibre APD (Fig. 6). 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. 6). 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 fibres.

Because 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 forskolin to activate adenylcyclase and increase intracellular cAMP. Forskolin (1 μM) alone ($n = 17$)

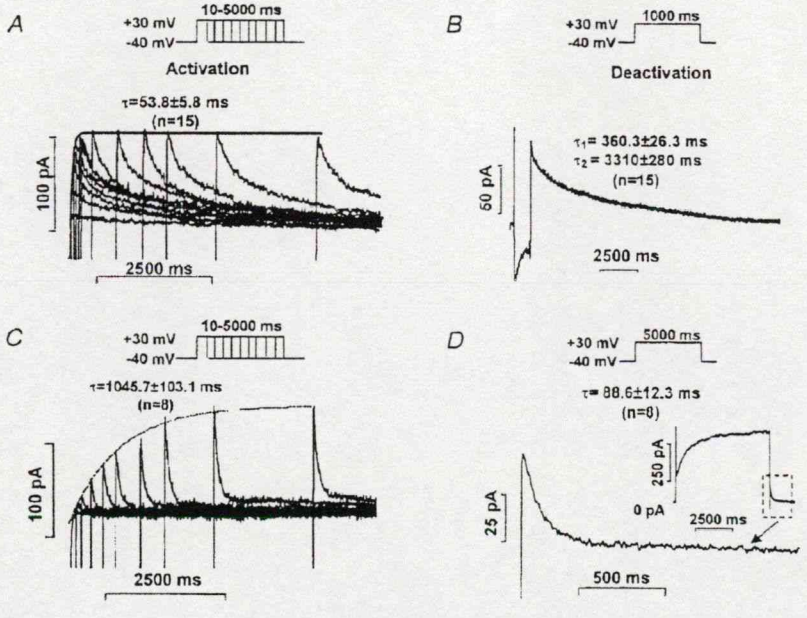

Figure 7. Activation and deactivation kinetics of I_{Kr} and I_{Ks} in dog ventricular myocytes
A and *C*, activation kinetics of I_{Kr} and I_{Ks}, respectively, measured as tail currents at −40 mV after test pulses to +30 mV with duration gradually increasing between 10 and 5000 ms. *B* and *D*, deactivation kinetics of I_{Kr} and I_{Ks} outward tail current, respectively, at −40 mV after a 1000 or 5000 ms, respectively, long test pulse to +30 mV. The inset in *D* shows I_{Ks} tail current at higher resolution.

markedly shortened APD in dog right papillary muscle paced at cycle lengths ranging between 300 and 5000 ms (i.e. from 190·2 ± 4·4 to 157·1 ± 3·3 ms and 258·2 ± 5·7 to 212·5 ± 4·2 ms at cycle lengths of 300 and 5000 ms, respectively). Addition of L-735,821 (100 nM) or chromanol 293B (10 μM) in the continuous presence of forskolin had little effect on APD (150·2 ± 2·2 *versus* 153·2 ± 2·6 ms and 207·5 ± 3·4 *versus* 209·0 ± 4·5 ms following L-735,821 and 164·1 ± 4·3 *versus* 176·0 ± 4·2 ms and 217·6 ± 5·1 *versus* 234·9 ± 9·1 ms following chromanol 293B at pacing cycle lengths of 300 and 5000 ms, respectively). These results again show that selective I_{Ks} block only slightly lengthened APD over a wide range of stimulation frequencies, even in the presence of elevated intracellular cAMP.

Estimation of I_{Ks} and I_{Kr} activation during the plateau phase of the action potential

Earlier results suggested that I_{Kr} activates rapidly during action potentials but deactivates slowly, while I_{Ks} activates slowly at more positive potentials (Gintant, 1996). In addition, I_{Ks} accumulation over successive depolarization is not likely since its deactivation is fast with respect to diastolic intervals occurring at physiological heart rates. 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. To examine this phenomenon further, we carefully evaluated the kinetics of I_{Kr} and I_{Ks} at depolarized potentials (+30 mV) corresponding to the action potential plateau.

In our study, I_{Kr} indeed activated rapidly in dog ventricular myocytes (Fig. 7*A*). Using gradually increasing test pulse durations from a holding potential of −40 mV to +30 mV in the presence of 100 nM L-735,821 to block I_{Ks}, the activation time constant (τ) for I_{Kr} was 53·8 ± 5·8 ms ($n = 15$) with an amplitude (A) of 69·7 ± 6·4 pA ($n = 15$). Deactivation of I_{Kr} on return to −40 mV from +30 mV was slow (Fig. 7*B*), and it was best fitted by a double exponential relation where the parameters were: $\tau_1 = 360·3 \pm 26·3$ ms;

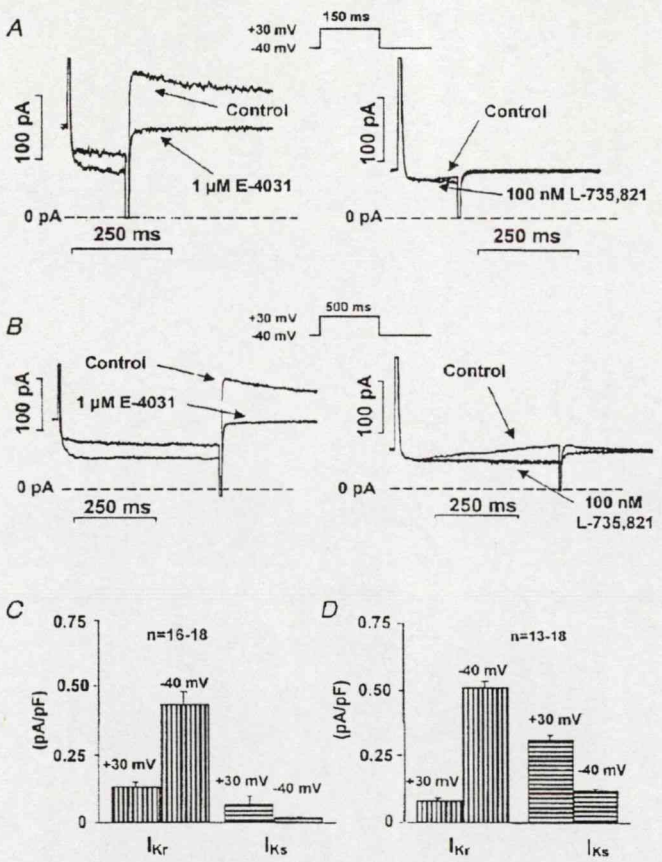

Figure 8. Comparison of the magnitude of I_{Kr} and I_{Ks} after short and long voltage pulses

A, recordings of E-4031 (I_{Kr}, left)- and L-735,821 (I_{Ks}, right)-sensitive currents after application of a short (150 ms) depolarizing test pulse to +30 mV from a holding potential of −40 mV. *B*, recordings of E-4031 (I_{Kr}, left)- and L-735,821 (I_{Ks}, right)-sensitive currents after a long (500 ms) depolarizing test pulse to +30 mV from a holding potential of −40 mV. *C*, average I_{Kr} and I_{Ks} currents at the end of a short (150 ms, right panel) and a long (500 ms, left panel) depolarizing test pulse to +30 mV, and peak tail current at −40 mV. Bars represent S.E.M.

$\tau_2 = 3310 \pm 280$ ms; $A_1 = 31{\cdot}8 \pm 0{\cdot}7$ pA and $A_2 = 34{\cdot}1 \pm 3{\cdot}14$ pA ($n = 15$).

I_{Ks} kinetics were also assessed but in the presence of 5 μM E-4031 to eliminate I_{Kr}. I_{Ks} activation under these conditions in dog ventricular myocytes was slow (Fig. 7*C*) ($\tau = 1045{\cdot}7 \pm 103{\cdot}1$ ms, $A = 61{\cdot}1 \pm 8{\cdot}3$ pA, $n = 8$). I_{Ks} deactivation in these myocytes was fast (Fig. 7*D*) ($\tau = 88{\cdot}6 \pm 12{\cdot}3$ ms, $n = 8$).

To estimate the magnitude of I_{Ks} and I_{Kr} activated during the cardiac action potential, we compared the amplitudes of the L-735,821-sensitive (I_{Ks}) and E-4031-sensitive (I_{Kr}) currents at the end of a 150 ms long test pulse to +30 mV and their tail currents on return to −40 mV. Using this protocol we assessed I_{Kr} and I_{Ks} at voltages corresponding to the plateau and repolarization phases of the action potential. Because deactivation of I_{Kr} is slow in comparison to its recovery from inactivation (Spector *et al.* 1996), tail currents measured at −40 mV do not accurately reflect the magnitude of I_{Kr} activated during the test pulse. The opposite situation may be expected with I_{Ks} because this current does not appear to inactivate and the driving force for K^+ is larger at positive than at negative voltages. We, therefore, measured I_{Ks} and I_{Kr} by subtracting membrane currents before and after 4–5 min of exposure to L-735,821 and E-4031, respectively. The E-4031-sensitive current (I_{Kr}) amplitude at the end of the 150 ms long test pulse was $25{\cdot}8 \pm 3{\cdot}2$ pA ($n = 16$), or about 29% ($86{\cdot}5 \pm 10{\cdot}5$ pA, $n = 16$) of the tail current amplitude measured after the test pulse returned to −40 mV (Fig. 8*A*, left panel). The L-735,821-sensitive current (I_{Ks}) during the test pulse to +30 mV was larger than its tail current on return to −40 mV (Fig. 8*A*, right panel). The magnitude of I_{Ks} tail current during the test pulse was $12{\cdot}5 \pm 0{\cdot}8$ pA at +30 mV *versus* $3{\cdot}5 \pm 0{\cdot}5$ pA at −40 mV ($n = 18$) still approximately an order of magnitude less than the I_{Kr} tail current.

We also compared I_{Ks} and I_{Kr} magnitudes during 'action-potential-like' test pulses. These test pulses were obtained by digitizing representative right ventricular dog action

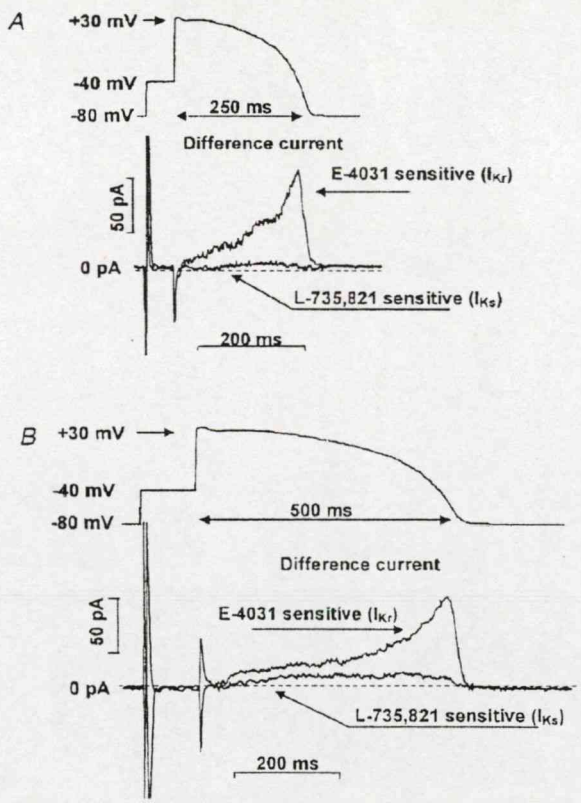

Figure 9. E-4031 (I_{Kr})- and L-735,821 (I_{Ks})-sensitive difference currents during short and long 'action potential-like' test pulse

A, E-4031-sensitive (1 μM) (I_{Kr}) and L-735,821-sensitive (100 nM) (I_{Ks}) difference currents recorded during an 'action-potential-like' test pulse in canine ventricular myocytes. The 'action-potential-like' test pulse was obtained by recording a normal canine ventricular action potential with a conventional microelectrode in a multicellular papillary muscle preparation and adding a 50 ms prepulse from −80 to −40 mV. *B*, recordings of E-4031-sensitive (I_{Kr}) and L-735,821-sensitive (I_{Ks}) currents when the action-potential-like test pulse duration was increased by a factor of 2 (i.e. to ~500 ms). Recordings in *A* and *B* were obtained in the same myocyte. Similar results to those illustrated were obtained in 4–7 additional myocytes.

Table 1. The effect of I.V. 1 mg kg^{-1} chromanol 293B and I.V. 1 mg kg^{-1} D-sotalol on the ECG interval durations in intact anaesthetized dogs

Interval durations	Chromanol 293B ($n = 6$)		D-Sotalol ($n = 5$)	
	Control	Chromanol 293B	Control	D-Sotalol
PP (ms)	463·3 ± 52·2	463·3 ± 39·4	360 ± 21·9	450·0 ± 19·5*
PQ (ms)	98·3 ± 10·5	95·0 ± 8·8	92·0 ± 10·2	96·0 ± 7·5
QRS (ms)	45·0 ± 2·2	48·3 ± 4·8	38·0 ± 2·0	42·0 ± 3·7
QT (ms)	223·3 ± 12·0	223·3 ± 9·5	194·0 ± 7·5	232·0 ± 3·7*
QTc (ms)	332·9 ± 16·1	330·5 ± 11·2	323·9 ± 7·3	346·5 ± 6·4*

QTc, QT was corrected using the Bazett equation. * $P < 0·05$.

potentials recorded with conventional microelectrodes. A 40 ms long prepulse to −40 mV was added at the beginning of the idealized action-potential-like test pulse (Fig. 9*A*). Under these conditions the I_{Kr} difference current (i.e. the E-4031-sensitive current) during the action potential plateau phase was small with its magnitude increasing as the test voltage became more negative (Fig. 9*A*). In contrast, the I_{Ks} difference current (i.e. the L-735,821-sensitive current) remained small throughout all phases of the action potential-like test pulse (Fig. 9*A*). These results indicate

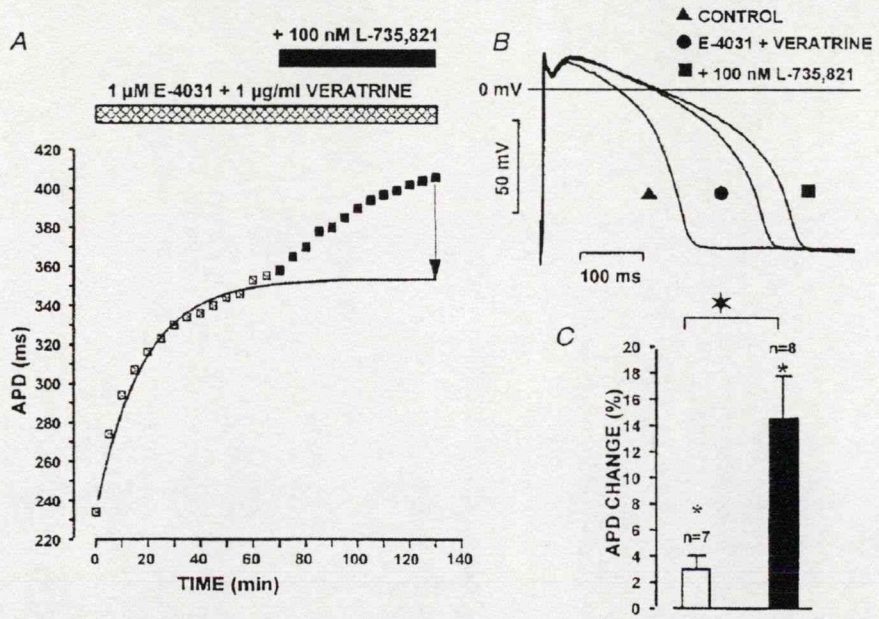

Figure 10. Effect of 100 nM L-735,821 on dog ventricular action potentials recorded in the presence of 1 μM E-4031 and 1 μg ml^{-1} veratrine

A, the time course of a representative experiment. At 0 min 1 μM E-4031 and 1 μg ml^{-1} veratrine were added and measurements were taken every 5 min until a 'quasi' steady state was achieved. Then 100 nM 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 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 (continuous line) so that the magnitude of its effect at 140 min is indicated by the arrow. *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). *C*, comparison of the effect of L-735,821 on 'short' (open bar) and on 'long' (filled bar) 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). The filled star represents significant changes between the bars ($P < 0·01$ in both cases). Columns and error bars indicate means and S.E.M.

that the outward current carried through I_{Kr} channels during the action potential is more than 10 times greater than through I_{Ks} channels (Figs 8*A* and 9*A*). These results agree well with the failure to increase ventricular and Purkinje fibre 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 action potential duration was artificially increased. In these experiments we either applied long (500 ms) step-wise, rectangular test pulses to +30 mV (Fig. 8*B*) or an 'action-potential-like' test pulse having a duration of 500 ms (Fig. 9*B*). I_{Kr} was not substantially changed due to its fast activation when the duration of the test pulse was increased from 150 to 500 ms (I_{Kr} magnitude was $21{\cdot}3 \pm 1{\cdot}9$ pA at +30 mV and $101{\cdot}0 \pm 14{\cdot}7$ pA at −40 mV, $n = 13$) (Fig. 8*B*, left panel, and Fig. 9*B*). However, the magnitude of I_{Ks} was significantly increased to $61{\cdot}5 \pm 5{\cdot}7$ pA at +30 mV and $22{\cdot}7 \pm 2{\cdot}5$ pA ($n = 18$) when test pulse durations were increased to 500 ms (Fig. 8*B*, right panel, and Fig. 9*B*).

The effects of L-735,821 and chromanol 293B on pharmacologically lengthened action potentials

The effects of both L-735,821 and chromanol 293B were tested in dog ventricular papillary muscle action potentials, lengthened pharmacologically by exposure to 1 μM E-4031 (to block I_{Kr}) and 1 μg ml^{-1} veratrine (a recognized sodium channel agonist). In these experiments, performed while continuously pacing at 1 Hz, recordings were taken every 5 min after initiating superfusion with 1 μM E-4031 + 1 μg ml^{-1} veratrine until a 'quasi' steady-state was attained (Fig. 10*A* and *B*). 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-735,821 or 10 μM chromanol 293B. L-735,821 markedly lengthened APD under these conditions from $383{\cdot}5 \pm 25{\cdot}2$ to $442{\cdot}1 \pm 32{\cdot}3$ ms ($P < 0{\cdot}01$, $n = 7$) (Fig. 10). This effect was in sharp contrast to the negligible effect of L-735,821 on normal APD (Figs 4 and 6). Comparable effects on APD were obtained with chromanol 293B in the continuous presence of E-4031 and veratrine (APD was $366{\cdot}1 \pm 13{\cdot}1$ ms before chromanol 293B *versus* $429{\cdot}5 \pm 23{\cdot}5$ ms after its addition, $P < 0{\cdot}01$, $n = 8$). These results indicate that the effect of I_{Ks} on APD is substantially increased when APD is abnormally lengthened.

Influence of I_{Ks} and I_{Kr} inhibitions on the QTc interval in anaesthetized closed chest dogs

To examine further whether I_{Ks} block lengthens APD and increases QT interval, we examined the effects of chromanol 293B (1 mg kg^{-1} I.V.) and D-sotalol (1 mg kg^{-1} I.V.) in anaesthetized dogs (Table 1). In agreement with our *in vitro* observations, chromanol 293B did not significantly affect QTc interval, while D-sotalol markedly lengthened it.

DISCUSSION

Summary of the main results

Our results indicate that both chromanol 293B and L-735,821, purportedly selective I_{Ks} blockers, did not substantially lengthen APD in either dog right ventricular papillary muscle or Purkinje fibre preparations. Equivalent concentrations of both compounds, however, substantially blocked I_{Ks} in isolated dog ventricular myocytes. Adenylcyclase stimulation by forskolin, known to increase I_{Ks} (Walsh *et al.* 1989), did not substantially enhance the small increase in APD induced by either chromanol 293B or L-735,821 in dog papillary muscle. In contrast, E-4031 and D-sotalol (recognized I_{Kr} blockers) markedly lengthened dog ventricular muscle and Purkinje fibre APD. In agreement with these *in vitro* results, QTc was increased *in vivo* by D-sotalol but not by chromanol 293B in anaesthetized 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.

Choice of drug concentrations

The concentrations of drugs used in this study are comparable to those previously described in the literature (Lathrop, 1985; Sanguinetti & Jurkiewicz, 1990; Salata *et al.* 1996*b*; Busch *et al.* 1996). D-Sotalol at a concentration of 30 μM, inhibited I_{Kr} by 30–50%; 1 and 5 μM E-4031 caused complete block. This amount of I_{Kr} block made examination of the effects of D-sotalol and E-4031 on Purkinje fibre APD difficult. E-4031-induced Purkinje fibre APD lengthening was, for example, so pronounced that recordings at pacing cycle lengths shorter than 1000 ms could not be achieved because these stimuli fell within the total refractory period.

The concentrations of L-735,821 (100 nM) and chromanol 293B (10 μM and 30 μM) were also comparable to those used by others (Salata *et al.* 1996*b*; Busch *et al.* 1996). This L-735,821 concentration completely blocked I_{Ks} as previously reported (Salata *et al.* 1996*b*). Chromanol 293B at 10 μM blocked I_{Ks} by 70% in agreement with findings in guinea-pig ventricular myocytes (Busch *et al.* 1996). Higher chromanol 293B concentrations, however, notably affected other repolarizing currents (Bosch *et al.* 1998). Although the application of a higher concentration of chromanol 293B made interpretation of its effect on action potential repolarization uncertain at best; we used 30 μM chromanol 293B in order to block I_{Ks} completely, during the assessment of I_{Kr}. At this chromanol 293B concentration, we observed marked I_{to} depression in good agreement with earlier reports from Bosch *et al.* (1998). High chromanol 293B concentrations may also block I_{Kr}; however, the results of this current study do not address or confirm this speculation.

The 1 mg kg^{-1} I.V. dose of chromanol 293B in the *in vivo* experiments was chosen because both chromanol 293B and

D-sotalol have similar molecular weights (324·4 *versus* 309, respectively) and both compounds were assumed to have similar potencies for channel block. Although chromanol 293B proved to be a potent I_{Ks} blocker in the patch-clamp measurements, the possibility that the applied dose of 1 mg kg^{-1} I.V. chromanol 293B did not completely block I_{Ks} cannot be ruled out.

Comparison of the results with earlier findings

I_{Ks} and I_{Kr} are both generally accepted as having important roles during normal cardiac action potential repolarization (Sanguinetti & Jurkiewicz, 1990; D. W. Liu & Antzelevitch, 1995; Singh, 1998). However, selective I_{Ks} blockers have only recently been available (Salata *et al.* 1996*b*; Busch *et al.* 1996). With the development of such I_{Ks} blockers, it is possible to determine directly the effect of I_{Ks} on APD.

The few published studies that have examined the effect of I_{Ks} on cardiac APD were performed in guinea-pig papillary muscle (Schreieck *et al.* 1997) as well as in isolated guinea-pig and human ventricular myocytes (Bosch *et al.* 1998; Bryant *et al.* 1998) and in rabbit Purkinje cardiocytes (Cordeiro *et al.* 1998). The results obtained often contradict one another. Schreieck *et al.* (1997) for example using conventional microelectrodes, did not observe a significant APD increase after exposing multicellular guinea-pig papillary muscle preparations to 10 μM chromanol 293B. This lack of effect has been argued to result from the absence of adrenergic stimulation (Schreieck *et al.* 1997). In contrast, Bosch *et al.* (1998), 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, isolated myocytes show enormous beat-to-beat variability probably due to loss of electrotonic influences among electrically coupled myocytes or the run-down of currents affecting repolarization. Nevertheless, our results using conventional microelectrode recordings in dog papillary muscles agree, in part, with those of Schreieck *et al.* (1997) in guinea-pig papillary muscle; i.e. 10 μM 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.* (1997) did following isoproterenol exposure in guinea-pig. This deviation from the findings of Schreieck *et al.* (1997) might be due to species differences. Certainly I_{Ks} amplitude is relatively large in the guinea-pig (Sanguinetti & Jurkiewicz, 1990) compared with that in the dog and other species (Gintant, 1996). In addition, we applied 1 μM forskolin while Schreieck *et al.* (1997) used 100 nM isoproterenol to activate adenylcyclase. Because other currents are also modulated by cAMP (e.g. I_{Ca} and I_{Cl}) that also affect APD (Harvey & Hume, 1989), 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 fibre strands than previously reported by Cordeiro *et al.* (1998) using L-735,821 in four single, isolated rabbit cardiac Purkinje fibre cells. These investigators reported marked APD lengthening after superfusion with only 20 nM L-735,821. The reason for this discrepancy in findings is unknown. However, some investigators have suggested that because of its physical and/or chemical properties, L-735,821 poorly penetrates multicellular preparations but easily enters single myocytes (J. J. Salata, personal communication). Be that as it may, the action potential plateau voltage in the rabbit Purkinje fibre cells illustrated by Cordeiro *et al.* (1998, Fig. 9) is approximately −20 mV, while in the same study (Cordeiro *et al.* 1998, 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.* (1998) was due to I_{Ks} block.

The effects of D-sotalol and E-4031 on Purkinje fibre APD in our study are in excellent agreement with those previously published (Strauss *et al.* 1970; Lathrop, 1985; Varró *et al.* 1986; Sanguinetti & Jurkiewicz, 1990).

Estimation of the amount of I_{Ks} and I_{Kr} activated during the action potential

We estimated I_{Kr} and I_{Ks} during normal ventricular action potentials. Currents measured during and after 200 ms rectangular and artificial action-potential-like test pulses indicated that I_{Kr} is several times greater than I_{Ks}. Consistent with these findings, the recent papers of Hancox *et al.* (1998) and Zhou *et al.* (1998) have also confirmed that I_{Kr} plays a crucial role in the action potential repolarization under physiological conditions. On the other hand, our finding suggests that I_{Ks}, unlike I_{Kr}, plays little role during normal action potential repolarization. Such a conclusion is well supported by the negligible effect of I_{Ks} block on isolated ventricular muscle and Purkinje fibre APD as well as on intact dog QTc.

When the duration of the rectangular or action potential-like test pulse was increased, however, I_{Ks} was more fully activated. Thus, I_{Ks} is expected to limit excessive APD lengthening when repolarization is abnormally lengthened. This speculation is supported by our experiments where APD was substantially increased pharmacologically by augmenting inward (I_{Na}) and decreasing outward (I_{Kr}) currents (Fig. 10).

Potential significance of the results

Prior to this study, I_{Ks} was believed vital to normal cardiac action potential repolarization. As such, I_{Ks} was thought to control normal APD and refractoriness (D. W. Liu & Antzelevitch, 1995; Sanguinetti & Keating, 1997; Singh, 1998). In addition, based on experiments performed in guinea-pig ventricular myocytes, selective I_{Ks} block was believed to increase APD without producing the undesired, reverse use-dependent APD lengthening which is

characteristic of I_{Kr} block (Jurkiewicz & Sanguinetti, 1993). This expectation was based on the finding 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 (Gintant, 1996) and human ventricular myocytes (Jost *et al.* 1998), 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 (1993) into question. It is also notable that Heath & Terrar (1996*b*) have recently reported rather rapid deactivation of I_{Ks} also in guinea-pig myocytes. Our finding that I_{Ks} block does not remarkably increase APD in either normal dog ventricular muscle or Purkinje fibres over a wide range of pacing frequencies directly contradicts the Jurkiewicz & Sanguinetti (1993) hypothesis. Our findings, however, must be examined in perspective with other recent observations. Shimizu & Antzelevitch (1998) have, for example, recently reported that chromanol 293B lengthened APD in wedge-perfused canine left ventricular muscle preparations. In these experiments, 1–10 μM chromanol 293B produced only a slight increase in APD, as in the present study. However, in that study (Shimizu & Antzelevitch, 1998) chromanol 293B concentrations greater than 30 μM substantially increased APD. Such concentrations are greater than those required to block I_{Ks} fully, and these chromanol 293B concentrations probably affect other outward currents involved in the control of APD (Bosch *et al.* 1998).

I_{Ks} block in the presence of sympathetic stimulation is also believed to selectively prevent APD shortening associated with cAMP-dependent augmentation of I_{Ks} (Vanoli *et al.* 1995). Such an effect could potentially provide anti-arrhythmic benefit and represent an innovative approach to arrhythmia treatment. In support of this speculation L-768,673 (a structural analogue of L-735,821) provides anti-arrhythmic efficacy following coronary artery ligation and sympathetic stimulation (Billman *et al.* 1998*a*,*b*). Our results in the presence of forskolin do not support such a speculation. As such, additional research is needed to clarify the effect of sympathetic stimulation on I_{Ks} and their combined role in arrhythmogenesis.

Although I_{Ks} may have little role in normal action potential repolarization, it probably plays a vital role when cardiac APD is abnormally lengthened by other means (e.g. by reductions in I_{Kr} or I_{K1} or increases in I_{Na} or I_{Ca}). 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 (Surawicz, 1989) and increase propensity for development of early afterdepolarization (El-Sherif, 1992) associated with Torsade de Pointes induction. Such a role for I_{Ks} in limiting excessive APD lengthening was first postulated by Ito & Surawicz (1981), and if I_{Ks} plays such a role, anti-arrhythmic agents producing non-selective block of I_{Kr} and I_{Ks} (e.g. quinidine and azimilide) might be associated with a greater pro-arrhythmic risk than 'pure' (selective) I_{Kr} blockers (e.g. sotalol and dofetilide). In agreement with this speculation, Salata *et al.* (1998) have recently recommended I_{Ks} activation for prevention of pro-arrhythmic complications due to excessive potassium channel block.

Some forms of inherited long QT syndrome (LQT) probably represent situations where loss of the protective effect of I_{Ks} is detrimental. For example, LQT1 is an inherited disorder where fewer I_{Ks} channels are expressed than in normal individuals. Our results in dog indicating that I_{Ks} plays little role in normal action potential repolarization suggest that its absence alone would not result in a prolonged APD and a long QT interval. Thus, with the presence of the LQT1 phenotype in man associated with reduced I_{Ks} expression it is difficult to reconcile our findings. This discrepancy between observations may have two explanations: (1) I_{Ks} is more abundantly expressed in man than in dog, or (2) reduction in I_{Ks} in both dog and man increases the likelihood that reduction in other outward currents (or an increase in inward current) results in LQT. Preliminary results in man showing that I_{Ks} is similar to that in dog and that its block does not affect normal papillary muscle APD (Varró *et al.* 1999) supports the second possibility. Thus, it may be that the absence of I_{Ks} in these individuals simply limits their ability to restrict excessive APD lengthening due to other causes (e.g. hypokalaemia or bradycardia). This explanation would account for the recent finding that the penetrance of genetic defects involving reduction in I_{Ks} channel expression (LQT1) is rather low compared with other forms of LQT (Swan *et al.* 1998; Priori *et al.* 1998). Some of these authors report that only about 25% of patients with genetic defects encoding for I_{Ks} channels actually had abnormally long QT intervals (Priori *et al.* 1998).

Marked gender differences have recently been described in the prevalence of inherited and acquired LQT that may be due to differences in potassium current expression (X. K. Liu *et al.* 1998). This is an important area of research interest and significant differences may exist in I_{Ks} expression in males and females. However, in the present studies no attempt was made to differentiate between results obtained in myocytes or preparations isolated from animals of different gender.

D. W. Liu & Antzelevitch (1995) showed in isolated dog ventricular myocytes that M cells express a lower density of I_{Ks} channels than do subendocardial or subepicardial cells. These investigators postulate on this basis 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 lengthen APD substantially 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 probably account for the differences in M cell and subendocardial ventricular muscle cell action potential configurations. Differences in endocardial and M cell sodium window currents (or slowly inactivating I_{Na}) density, for example, may help account for APD differences in these two cell types.

Conclusions

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 mechanisms.

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ABSTRACTS

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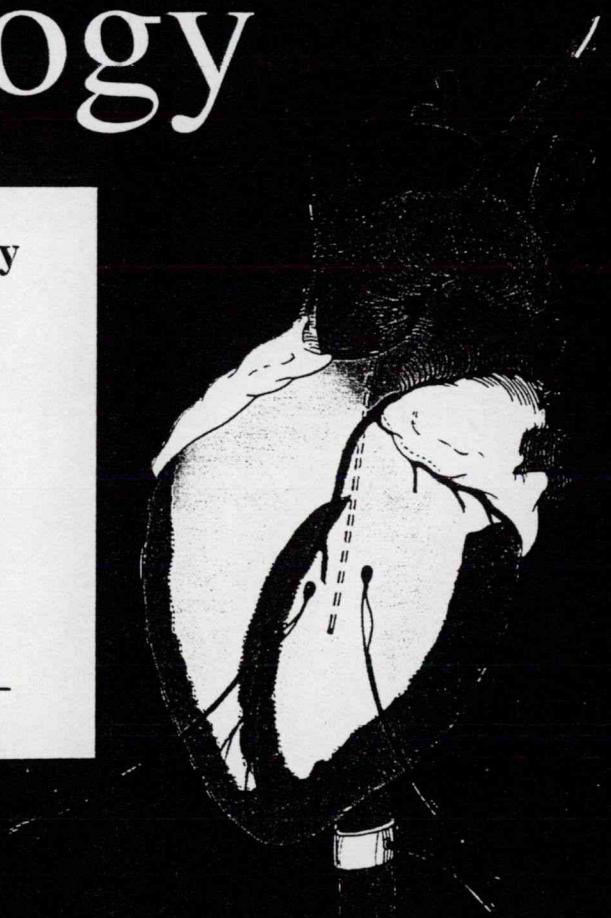

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THE CELLULAR ELECTROPHYSIOLOGICAL EFFECTS OF DESETHYLAMIODARONE IN DOG CARDIAC VENTRICULAR MUSCLE AND PURKINJE FIBERS

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Amiodarone, which has unique and complex mode of action, is one of the most effective antiarrhythmic compounds used to treat cardiac rhythm disturbances. After chronic treatment one of its active metabolite, desethylamiodarone appears in the plasma and tissue. The cardiac electrophysiological effects of desethylamiodarone have however not been entirely elucidated. Therefore, we have investigated the cellular electrophysiological effect of 10 μM desethylamiodarone in dog ventricular muscle and Purkinje fibers by applying the standard microelectrode technique. In Purkinje fibers, 10 μM desethylamiodarone induced use-dependent depression of the maximal rate of depolarization (V_{max}) [at stimulation cycle length (CL) of 400 ms = -28.4$\pm$6.6 %, mean$\pm$S.E., n=4, $p<0.05$] with a recovery time constant of 341.85$\pm$81.6 ms; (n=4). At CL of 1000 ms desethylamiodarone shortened action potential duration from 413.2$\pm$30.5 ms to 362.0$\pm$28.1 ms (n=4, $p<0.05$) and did not influence significantly the maximal diastolic potential and the action potential amplitude. In ventricular muscle, 10 μM desethylamiodarone caused moderate, use-dependent depression of V_{max} (at CL of 400 ms = -14.3$\pm$9.4 %; n=5) without changing action potential duration and other electrophysiological parameters. These results indicate that the electrophysiological effects of desethylamiodarone are different in cardiac Purkinje and ventricular muscle fibers and similar to those observed earlier with amiodarone. The work was supported by OTKA T 016651 Grant.

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COMPARISON OF THE CELLULAR ELECTROPHYSIOLOGIGAL EFFECTS OF AMIODARONE AND DRONEDARONE IN CANINE VENTRICULAR MUSCLE AND PURKINJE FIBERS

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It is generally accepted that at present, amiodarone is one of the most effective antiarrhythmic drugs to treat ventricular tachyarrhythmias. Amiodarone had originally been classified as a Class III antiarrhythmic drug, but later it became evident that in additions to lengthening cardiac repolarization, it exerts other important cellular electrophysiological effects as well, which may explain its powerful antiarrhythmic potency. Since amiodarone causes various extracardiac side effects which limit its usefulness, great effort was made to develop new compounds with similar electrophysiological profile, lacking side effects. Dronedarone (SR-33589), which chemically resembles amiodarone is such a compound; it is effective in various in vivo experimental arrhythmia models. The purpose of the present study was to characterise the cellular electrophysiological effects of dronedarone, and to compare it with those of amiodarone. The experiments were carried out in canine papillary muscle and Purkinje fibers by applying the conventional microelectrode technique. Dronedarone (10 μM), like amiodarone (10 μM), did not significantly change the action potential duration (APD) at 1Hz stimulation frequency, but significantly shortened APD 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 I/B type V_{max} block and suppressed early after- depolarization induced by dofetilide + $BaCl_2$ in Purkinje fibers. It was concluded that after acute application, dronedarone exerts similar cellular electrophysiological effects to those of amiodarone, therefore it is a promising candidate for future development.