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Original Article

Rabbit ventricular myocardium undergoing simulated ischemia and reperfusion in a double compartment tissue bath: a model to investigate both antiarrhythmic and arrhythmogenic likelihood

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Abstract: An ischemia/reperfusion-simulating model in rabbit tissue should be right oriented and clinically relevant to provide a non expensive approach for manipulations of currents involved in the repolarization process. Standard right ventricular guinea-pig (N=18) and newly investigated rabbit (N=12) myocardial strips were placed in a special perfusion chamber allowing partition into two segments independently superfused with oxygenated Tyrode's solution or a modified Tyrode's solution mimicking ischemia by: 1) increased extracellular potassium concentration (12 mmol/L), 2) decreased HCO_3^- concentration (9 mmol/L), leading to a decrease in pH (6.90 ± 0.05), 3) decreased pO_2 by replacement of 95% O_2 and 5% CO_2 by 95% N_2 and 5% CO_2 gas mixture, and 4) complete withdrawal of glucose. There were significant differences in rabbit as compared to guinea-pig preparations in baseline ($p<0.02$) and post-ischemic-like ($p<0.01$) APA and RMP with lower values in the formers, and lower post-ischemic Vmax in rabbit preparations (25±15 versus 97±83 V/s, $p<0.01$) but neither baseline nor post-ischemic-like or absolute changes in APD_{50}, APD_{90} were different. In ischemia- and reperfusion-like phases, there were high proportions of single spontaneous repetitive responses, both in guinea-pig (respectively 50 and 89%) and rabbit preparations (respectively 67 and 92%). Guinea-pig preparations showed higher incidence of severe spontaneous repetitive responses (61 versus 17%, $p<0.02$). This rabbit model is proposed to investigate both anti- and pro-arrhythmic effects of drugs acting at various levels electrophysiologically, which may be obtained with great power and relatively few (around 10 per group) preparations. This model should now be tested pharmacologically.

Keywords: Electrophysiology in vitro, action potentials, repetitive responses, rabbit, guine-pig, ventricular myocardium, simulated ischemia

Introduction

Guinea-pig right ventricular myocardium submitted to simulated ischemia in a double compartment tissue bath enabled *in vitro* studies of the antiarrhythmic potential of different compounds [1-5] by studying a portion superfused with normal oxygenated Tyrode's solution as compared to the same adjacent part receiving an ischemia-mimicking superfusion. The fundamental idea was that there was the possibility to approach, in a softer model than using anesthetized open-chest large animals [6-8], the myocardial post-ligation border zone facing normal and abnormal coronary perfusion [9]. A critical area was therefore closely investigated whereby arrhythmias may originate and electrophysiological data provided insights into antiarrhythmic drug actions[1-8] and the multivariate prediction of spontaneous repetitive responses [1, 10].

Rabbit ventricular tissues should represent a more closely human-resembling *in vitro* approach to arrhythmogenesis since [11] the ratio of rapid to slow outward repolarization currents (I_{Kr}/I_{Ks}) is higher in rabbits [12, 13] (around 13) than in guinea-pigs [12, 13] (around 0.1)

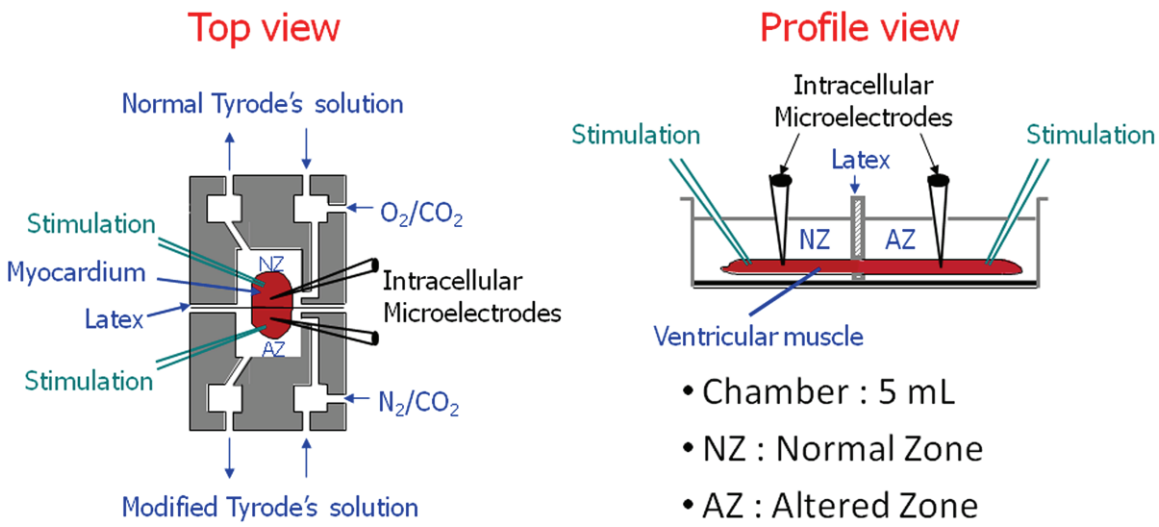

Figure 1. Top and profile views of the double compartment tissue bath used for the experiments. The volume chamber of 5 ml enabled the right ventricular strips (slightly longer in rabbits) to be gently passed under the latex membrane in order to have one portion superfused with normal Tyrode's and the adjacent portion with the altered, ischemia-simulating solution.

while being much similar to humans [12] (between 3 and 6). However, although normoxic endocardial tissue fragments (10 mm long, 5 mm wide, 1.5 mm thick), containing free-running Purkinje fibres dissected from rabbit left ventricular endocardium, near the septum, were studied electrophysiologically [11, 14], thinner rabbit right ventricular strips submitted to simulated ischemia and reperfusion were never investigated.

The aim of the study was to compare standard right ventricular myocardium tissues from guinea-pigs and a newly designed rabbit model. Tissues underwent oxygenated versus altered superfusions with simulated ischemia and reperfusion and the electrophysiological changes between normal and altered zones were investigated along with the incidences of spontaneous repetitive responses. In spite of much similar electrophysiological changes during simulated ischemia, the rabbit model showed seemingly high incidences of single repetitive responses and significantly lower incidence of more serious responses. Thus, both antiarrhythmic and arrhythmogenic potentials might be addressed in the rabbit model which indicates a great potential for pharmacological investigations.

Methods

Care of the animal conformed to the recommendations of the Helsinki Declaration, and the study was performed in accordance with the regulations of the official edict of the French Ministry of Agriculture.

Material

Guinea-pigs and rabbits of either sex (respectively weighing 300 to 400 and 1500 to 2000 g) were euthanized under anesthesia with sodium pentobarbital 125 mg/kg i.p. The hearts were quickly removed and standard strips of right ventricular myocardium (approximately 8 mm wide and 16 mm long in the guinea pig and approximately 8 mm wide and 20 mm in the rabbit) were dissected from the free wall and placed (**Figure 1**) in a special perfusion chamber bath (volume of 5 ml) separated into 2 compartments by a thin latex membrane [1-5]. This latex membrane is perforated at its bottom, allowing the ventricular strip to be passed through and therefore be divided into two zones, called the Normal Zone (NZ) and the Altered Zone (AZ) respectively. This double compartment partition allowed the two segments of the same ventricular strip to be independently superfused at a rate of 3 ml/min. The absence of leak under the latex membrane between the two compartments was tested at the end of each experiment by means of dye injection (methylene blue) into one of the compartments. The ventricular strip was pinned, endocardial surface upward, on the silicon base of the bath. The right ventricular base,

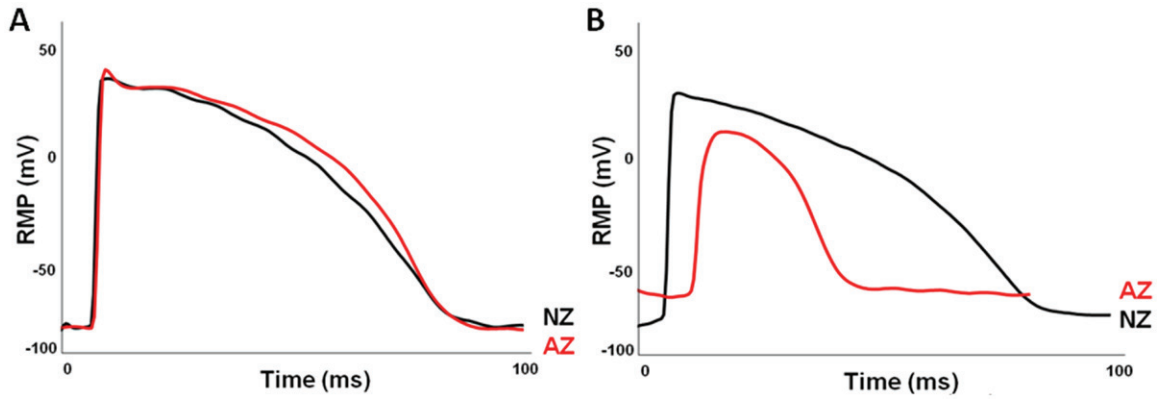

Figure 2. At baseline, action potentials recorded in right ventricular rabbit myocardium were practically identical (panel A) in normal (NZ) and altered (AZ) compartments, respectively. After 30 min of simulating ischemia superfusion, the AZ action potential was greatly modified (panel B): these changes, typical of the ischemic-like action potential, were similar to those seen in guinea-pig right ventricular tissues [1-5, 10]. RMP: resting membrane potential.

near the tricuspid valve, was constantly placed in the NZ compartment. Temperature at the level of the double chamber, including that of incoming fluids, was controlled and maintained to 36.5 ± 0.5°C by a circulating thermostated bath (Polystat 5HP, Bioblock, France).

Superfusion solutions

Studies were performed in Tyrode's solution oxygenated with a mixture of 95% oxygen and 5% carbon dioxide (pO_2 and pCO_2 at 510 ± 20 and 34 ± 2 mmHg, respectively). The composition (mmol/L) of the reference standard Tyrode's solution was: Na^+, 135; K^+, 4; Ca^{2+}, 1.8; Mg^{2+}, 1.0; $H_2PO_4^-$, 1.8; HCO_3^-, 25; Cl^-, 117.8; and glucose, 11.0. The pH was maintained at 7.35 ± 0.05 (fitted with diluted HCl). Modified Tyrode's solution mimicking ischemia differed from the standard one by: 1) an increased extracellular potassium concentration (12 mmol/L), 2) a decreased HCO_3^- concentration (9 mmol/L), leading to a decrease in pH (6.90 ± 0.05), 3) a decrease in pO_2 by replacement of 95% O_2 and 5% CO_2 by 95% N_2 and 5% CO_2 gas mixture, and 4) a complete withdrawal of glucose. These modifications of Tyrode's solution have been shown to simulate [1-5, 10] *in vitro* the electrophysiological abnormalities induced *in vivo* by ischemia [9].

Data acquisition and analysis

The preparations were stimulated at a frequency of 1 Hz via bipolar Teflon-coated steel wire electrodes positioned near the two extremities of the ventricular strip either in the NZ or AZ. Stimulation was applied either in one or the other half of the muscle preparation with a home-built commutator. Stimuli were rectangular pulses, 2 ms in duration and twice the diastolic threshold intensity (around 2-2.5 V) delivered by a programmable stimulator (SMP-310, Biologic, France). During the protocol, stimulation was stopped whenever spontaneous repetitive responses occurred.

Transmembrane action potentials were recorded simultaneously in both ventricular regions (**Figure 2A**) by use of intracellular glass microelectrodes filled with KCl 3M (tip resistance 10 to 30 MΩ) coupled to Ag/AgCl microelectrode holders leading to the double input stage of a high impedance capacitance-neutralizing amplifier. The two reference silver-silver chloride electrodes were positioned in the superfusate of each chamber, close to the preparation. Action potentials were monitored on a digital memory oscilloscope (Gould Instrument Systems Inc) and digitized by a system of cardiac AP automatic acquisition and processing device (DATAPAC, Biologic, France). The following action potential parameters were automatically recorded and measured: action potential amplitude (APA), resting membrane potential (RMP), action potential durations measured respectively at 50% and 90% of full repolarization (APD_{50} and APD_{90}) and maximal upstroke velocity of action potential (Vmax). Moreover, absolute changes of the abovementioned parameters were considered by applying the general formula: [(Xb) – (Xn)], where Xb and Xn

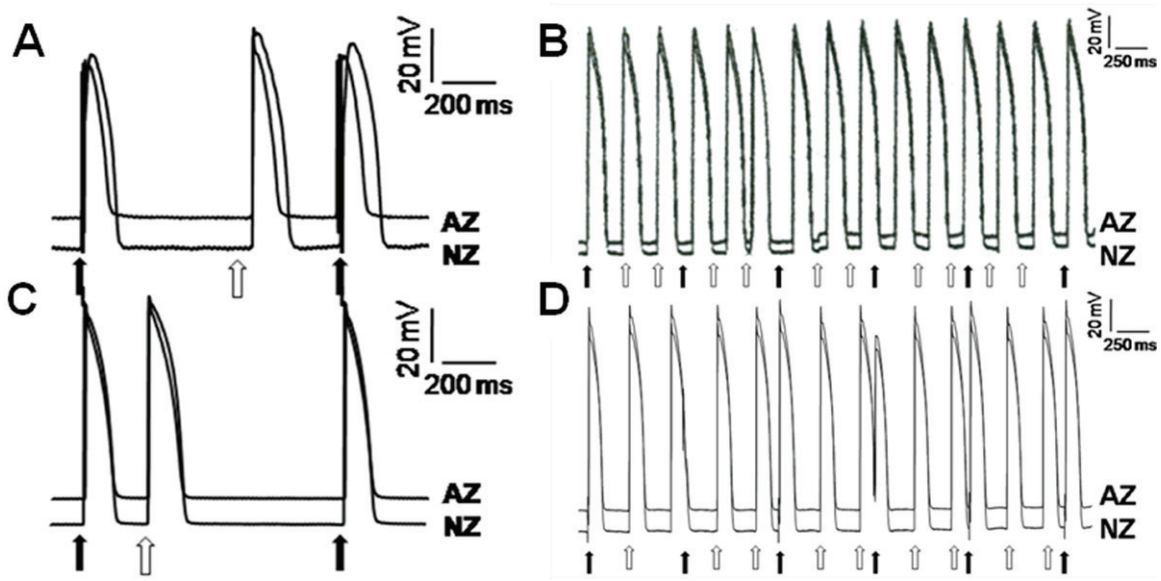

Figure 3. Single and severe (salvos of 10 or more consecutive) repetitive responses after simulated reperfusion in guinea-pig (respectively, panel A and B) and rabbit (respectively, panel C and D) preparations. Dark arrows indicate electrically driven complexes, whereas white arrows indicate spontaneous responses.

are individual electrophysiological parameters at baseline and after 30 min of simulated ischemia.

Experimental protocol

During a 120 min equilibration period, the two compartments were superfused with normal Tyrode's solution and the right ventricular muscle was stimulated at a frequency of 2 Hz. Thereafter, the preparation was stimulated at 1 Hz during the experiments and the AZ chamber was superfused during 30 min with the modified Tyrode's solution (simulated-ischemic period: **Figure 2B**) and then returned for 30 min to normal Tyrode's solution superfusion (simulated-reperfusion period), while the NZ compartment remained in normoxic conditions.

Both during simulated-ischemia and simulated-reperfusion, 3 types of spontaneous repetitive responses were recorded: (1) single premature ventricular contractions; (2) salvos (3 to 9 spontaneous action potentials) and (3) sustained (10 or more spontaneous action potentials) arrhythmia (**Figure 3**) as classified previously [1-5, 10]. Preparations showing electrical disturbances or arrhythmias before the onset of simulated ischemia were discarded.

Statistical analysis

Data were expressed as mean ± SD. Incidences of arrhythmias were calculated and treated as proportions. Analysis of variance (ANOVA) was used to test intergroup differences. The Fisher's exact test was used for comparison of nonparametric categorical data. Group sample sizes were considered (5 by 5 up to 50) and the powers were calculated for both anti- or pro-arrhythmic effects. For anti-arrhythmia and pro-arrhythmia cases the proportions in group one (the treatment group) were assumed to be respectively 0.9200 and 0.1700 under the null hypothesis and 0.2900 and 0.8000 under the alternative hypothesis. The proportions in group two (the control group) were respectively 0.9200 and 0.1700. The test statistic used was the two-sided Z test with pooled variance [15]. PASS 11 and NCSS version 2007 (by J Hintze, Kaysville, Utah; see www.ncss.com) were used. Differences were considered significant when $p<0.05$.

Results

Figure 1 shows top and profile views of the double compartment tissue bath used for the experiments. The volume chamber enabled the right ventricular strips (slightly longer in rabbits) to be carefully passed under the latex membrane in order to have one portion superfused with normal Tyrode's and the adjacent part of the same strip superfused with the altered, ischemia-simulating solution. At baseline, action potentials recorded in right ventricular

Table 1. Electrophysiological parameters and the incidences of spontaneous repetitive responses in preparations stimulated at 1 Hz

	Guinea pigs (N=18)	Rabbits (N=12)	F	p<
Baseline Electrophysiological parameters (in the NZ)				
Action potential amplitude (mV)	117±7 (106-127)	106±10 (94-122)	11.74	0.01
Resting membrane potential (-mV)	86±5 (97-76)	82±4 (87-76)	6.59	0.02
Action potential duration 50% (ms)	114±23 (52-147)	102±25 (46-143)	1.94	0.18
Action potential duration 90% (ms)	128±22 (75-171)	144±18 (116-177)	0.66	0.42
Maximal upstroke velocity (V/s)	231±78 (114-402)	279±58 (161-389)	3.36	0.08
Electrophysiological parameters after 30 min of simulated-ischemia (in the AZ)				
Action potential amplitude (mV)	82±22 (35-127)	60±10 (35-72)	9.98	0.01
Resting membrane potential (-mV)	63±8 (78-46)	53±7 (63-44)	11.61	0.01
Action potential duration 50% (ms)	48±23 (12-84)	42±11 (23-64)	0.69	0.41
Action potential duration 90% (ms)	64±23 (29-108)	56±11 (38-72)	1.25	0.27
Maximal upstroke velocity (V/s)	97±83 (2-276)	25±15 (1-57)	8.77	0.01
Differences from baseline after 30 min of simulated-ischemia (between NZ and AZ)				
Action potential amplitude (mV)	34±22 (-8-74)	45±16 (25-78)	2.15	0.15
Resting membrane potential (-mV)	25±14 (12-76)	28±9 (14-41)	0.16	0.69
Action potential duration 50% (ms)	66±29 (3-111)	59±25 (22-108)	0.41	0.53
Action potential duration 90% (ms)	74±30 (18-125)	88±22 (50-130)	1.94	0.18
Maximal upstroke velocity (V/s)	134±139 (-92-402)	254±61 (141-371)	7.64	0.01
Incidences of spontaneous repetitive responses on simulated-ischemia				
Single beats (%)	50	67	0.78	0.38
Salvos of 3-9 consecutive beats (%)	17	8	0.41	0.53
Salvos of 10 or more consecutive beats (%)	33	8	2.56	0.12
Incidences of spontaneous repetitive responses on simulated-reperfusion				
Single beats (%)	89	92	0.06	0.81
Salvos of 3-9 consecutive beats (%)	33	17	0.99	0.32
Salvos of 10 or more consecutive beats (%)	61	17	6.7	0.02

Data are mean ± standard deviation (and intervals) except incidences given as percent values and analyzed by chi square between groups (n/N). NZ=normal zone; AZ=altered zone.

rabbit myocardium were practically identical (**Figure 2A**) in normal (NZ) and altered (AZ) compartments, respectively. After 30 min of ischemic-like superfusion, the AZ action potential was greatly modified (**Figure 2B**): these changes were similar to those seen previously in guinea-pig right ventricular tissues [1-5, 10]. After simulated-reperfusion, obtained by superfusing the AZ with oxygenated Tyrode's solution, both guinea pig and rabbit preparations showed spontaneous repetitive responses, either single, frequent (3 to 9 consecutive beats) or severe (**Figure 3**).

Table 1 summarizes average and interval electrophysiological parameters at baseline and following the 30 min ischemic-like period in 18 guinea-pig and 12 rabbit preparations stimulated at 1 Hz. Absolute changes (from baseline to 30 min after ischemic-like superfusion) are also shown for APA, RMP, APD_{50}, APD_{90} and Vmax. There were slight differences in rabbit as compared to guinea-pig preparations in baseline ($p<0.02$) and post-ischemic-like ($p<0.01$) APA and RMP. Absolute Vmax change was higher in rabbit than in guinea-pig (254±61 versus 134±139V/s, $p<0.01$) preparations. It is probable that this follows to the significantly lower post-ischemic-like values in rabbit as compared to guinea-pig preparations (25±15 versus 97±83 V/s, $p<0.01$). On the other hand, neither baseline nor post-ischemic-like or absolute changes in APD_{50}, APD_{90} were significantly different between guinea-pig and rabbit preparations.

The incidences of spontaneous repetitive responses distributed according to their onset,

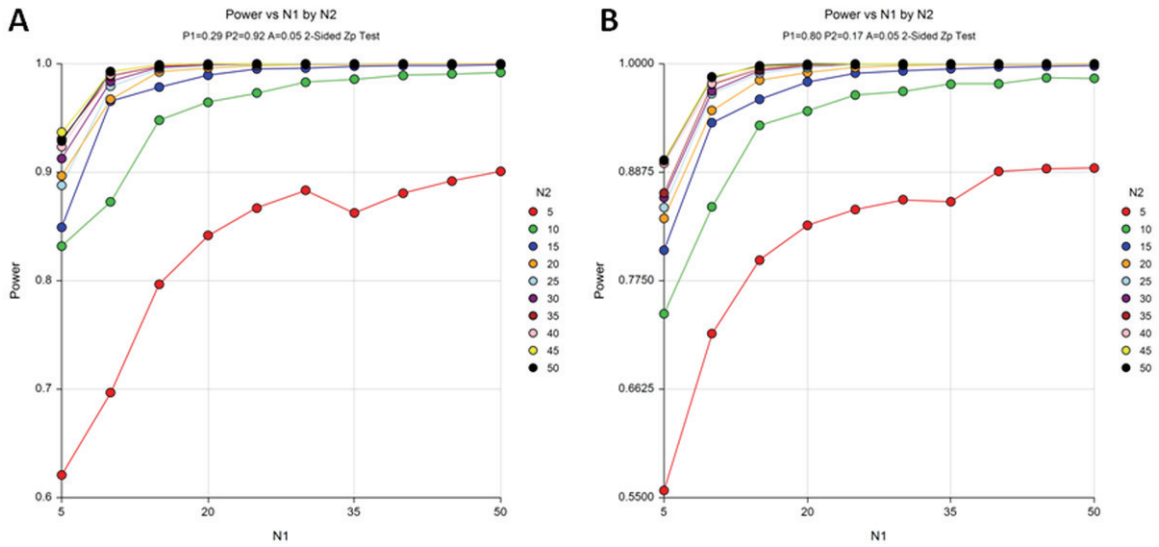

Figure 4. Anti-arrhythmia (panel A) and pro-arrhythmia (panel B) power calculations based on 5 by 5 incrementel experimental preparations (up to 50) defined following the observed incidences in the rabbit model (as shown in Table 1). Identical absolute delta proportions (63%) were selected. To obtain acceptably powered (>0.8) solutions, at least 10 preparations per group should be included. Thus, by selecting post-reperfusion-like single beats for anti-arrhythmic power and severe salvos for arrhythmogenic power in the rabbit model, correct conclusions are obtained with an acceptable number of experiments.

respectively during the ischemic-like or the reperfusion-like periods are also shown in **Table 1**. Average onset times of spontaneous repetitive responses were not statistically different between guinea-pig and rabbit preparations, respectively during the ischemic-like (1256±756 versus 810±853 s, p=0.14) or the reperfusion-like (2597±636 versus 2579±503 s, p=0.71) periods. Incidences of all spontaneous repetitive responses during simulated ischemia and of single and frequent types were comparable in guinea-pig and rabbit preparations. In ischemia- and reperfusion-like phases, there were high proportions of single spontaneous repetitive responses, both in guinea-pig (respectively 50 and 89%) and rabbit preparations (respectively 67 and 92%). A statistically significant difference was seen between guinea-pig and rabbit preparations in the incidence of severe spontaneous repetitive responses (salvos of 10 or more consecutive beats), much lower in the latter group (61 versus 17%, p<0.02).

Figure 4 illustrates 2 cases of anti- and pro-arrhythmia power calculations based on different numbers of experimental preparations defined on the observed incidences in the rabbit model (**Table 1**). In both cases an identical absolute delta proportion (63%) was selected. It is evident that in order to obtain acceptably powered (>0.8) solutions, at least 10 preparations per group should be included. Although these calculations vary [15] with different proportions and/or different absolute delta changes, **Figure 4** shows that the rabbit model, by selecting post-reperfusion-like single beats for anti-arrhythmic power and severe salvos for arrhythmogenic power, might enable statistically correct conclusions with an acceptable number of experiments.

Discussion

The rabbit model presented with similar incidences of single repetitive responses (**Figure 3**) as compared to the guinea-pig model (**Table 1**). In these circumstances, the anti-arrhythmic potential of drugs might be tested, as was the case with the guinea-pig model [1-4, 10], with high power and groups of 10 preparations (**Figure 4A**). The advantage with the rabbit model should be the potential to also test pro-arrhythmic effects of compounds, based on severe repetitive responses (**Figure 3**), in relatively short series with high power (**Figure 4B**). Therefore, the rabbit model of right ventricular myocardium partly submitted to normoxic and partly to ischemic- and reperfusion-like superfusions may be considered apt to investigate

both anti- and pro-arrhythmic effects of drugs acting at various levels electrophysiologically, which may be obtained with great power and relatively few preparations.

Our guinea-pig and rabbit preparations were very similar electrophysiologically (**Table 1**) and the most evident post-ischemia-like change was in Vmax. Interestingly, baseline action potential durations were similar between the two species (although longer in rabbits, 144±18 ms, than in guinea-pigs, 128±22 ms, F=0.66, p<0.42) and although absolute APD_{90} changes were larger in rabbits than in guinea-pigs (88±22 versus 74±30 ms, F=1.94, p<0.18), there was no statistically significant differences. It is possible that much larger series are needed to fully uncover the rabbit versus guinea-pig differences in APD that, at least theoretically, may make rabbit repolarisation longer and more representative of the human ventricular APD. Indeed, rabbit I_{Ks} kinetic properties [16] much resemble those of human cardiac myocytes [12, 17]. On the other hand, the ratio of rapid to slow outward repolarization currents (I_{Kr}/I_{Ks}) [11] is higher in rabbits [12, 13] than in guinea-pigs [12, 13] while being much similar to humans [12]. However, in extrapolating from different species, tissues or experimental approaches with various degrees of complexity [12, 18] there might be clear discrepancies in how different drugs exert their electrophysiological effects, which likely translate into considerable pro-arrhythmic differences, to the extent that results from one level of complexity (i.e. I_{HERG}) cannot forecast the results of the other level of complexity (i.e. whole tissue or whole organ) [12]. In addition, in whole heart experiments, both from rabbit and guinea-pigs, I_{Ks} blockade (by chromanol 293B or HMR 1556) was concluded as pro-arrhythmic with a variable degree of correlation with APD prolongation [19, 20] which we could not confirm in normoxic rabbit Purkinje fibres where I_{Ks} blockade was neutral whereas I_{Kr} blockade was pro-arrhythmic [11].

We have used in the past guinea-pig right ventricular tissues submitted to simulated ischemia and reperfusion to study the effects of sotalol [1], dofetilide [2, 4] and azimilide [3]. Although species and pathophysiology were different, I_{Kr} blockers had pro-arrhythmic effects similar to what seen in normoxic rabbit Purkinje fibres [11]. However, during simulated-ischemia other currents (i.e. I_{KATP}) may have come into play [1-4] to modulate and/or add to the effects of I_{Kr} blockers. Nevertheless, it is clear that d-sotalol and dofetilide may have a double-edge effect on border-zone arrhythmias [1, 2, 4] and the clinical counterpart was seen in SWORD [21] and DIAMOND [22] trials whereby an increased risk of life-threatening arrhythmias was observed in presence of ischemia. With azimilide, in normoxic rabbit Purkinje fibres [11] we observed that 100 nM and 500 nM induced similar APD prolongations as compared to d-sotalol (10 μM) and a significantly lower incidence of EAD in presence of epinephrine. Azimilide was the first non-selective agent able to block [23] both I_{Kr} and I_{Ks} and presented a ratio of I_{Kr} to I_{Ks} azimilide-induced block nearly 10 to 1 as detected in ferret papillary muscle by IC_{50} of 0.4 and 3 mM, respectively [24]. However, we showed previously [3], that a 30 min ischemia period produced less severe APD_{90} shortening in guinea-pig ventricular tissue exposed to a combination of dofetilide 10 nM and HMR 1556 than that produced on the same tissue exposed to a high dose of azimilide (500 nM), a difference not shown with lower dose (100 nM). Again the clinical counterpart may be seen in the results of the ALIVE trial whereby no antiarrhythmic effectiveness of non-selective I_K block was seen when ischemia was present [25].

Repolarizing currents are the target of class III compounds [1-5, 11] that, although not really effective against ventricular arrhythmias in presence of acute myocardial ischemia, prompted several large multicenter studies without clear-cut clinical impact [21, 22, 25]. Unfortunately, before undergoing clinical testing, sotalol, dofetilide and azimilide were not investigated in pertinent *in vitro* models where ischemia was taken into consideration. The selection of a set of pertinent models [12] whereby investigations on I_{Ks} activity or the combination of I_{Kr} and I_{Ks} activities might be performed [17, 19, 20] is indeed an open question. An ischemia/reperfusion-simulating model in rabbit right ventricular myocardium should be right oriented and clinically relevant [11-13, 17, 18]. It should provide a non expensive approach to investigate manipulations of essential currents involved in the repolarization process.

It is clear that the new rabbit model should now be used to test pharmacological interventions.

Not only electrophysiological parameters in both normal and ischemic-like zones [2] but also APD dispersion and the spontaneous or extrastimulus-related repetitive responses correlates [1, 26] should be studied since all these factors are important for establishing anti- and pro-arrhythmic risk in different conditions [27, 28]. As this model was shown to enable good power with an acceptable amount of experiments (**Figure 4**), to test it pharmacologically should be worthwhile.

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