AN ANALYSIS OF ANTIARRHYTHMIC ACTION IN A SERIES OF RELATED COMPOUNDS WITH REFERENCE TO THEIR SELECTIVITY FOR ISCHAEMIC TISSUE

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ABSTRACT

Treatment with antiarrhythmic drugs has been largely ineffective with respect to reducing mortality in patients with myocardial infarction due to the paradoxical tendency of some antiarrhythmic drugs to have concommitant proarrhythmic effects. This phenomenon may arise when electrophysiologic effects in normal myocardium occur at doses similar to those that suppress arrhythmogenic substrates in ischaemic tissue. This thesis examines the actions of six structurally-related antiarrhythmic drugs to test the hypothesis that drugs which act selectively in ischaemic myocardium will be more effective antiarrhythmic agents than drugs which lack such selectivity.

An isolated rat heart model was developed that allowed for simultaneous measurement of drug effects on optically-recorded action potentials in normal and ischaemic myocardium following coronary occlusion. This technique yielded quantitative indices of drug selectivity for ischaemic tissue with respect to effects on excitability (V_{max}) and repolarisation (APD). These results were compared to the antiarrhythmic profiles of each drug as determined in separate rat models of non-ischaemic and ischaemic arrhythmias. In the latter experiments slope and goodness of fit (r^2), which measure variability about the curve for antiarrhythmic protection, were interpreted as indices of selectivity for antiarrhythmic versus proarrhythmic effects.

Potency for protection against ischaemic arrhythmias was inversely related to slope and goodness of fit to the antiarrhythmic dose-response curve. However, lack of potency did not compromise efficacy. Thus, effective protection against ischaemic arrhythmias was better defined by steep slopes and strong goodness of fit coefficients since these drugs showed maximum safety and efficacy, even though they tended to be less potent. Slope and goodness of fit correlated strongly with increased potency for prolonging repolarisation in ischaemic versus normal tissue, demonstrating that drugs which act selectively in ischaemic myocardium are more ideal therapeutic antiarrhythmic agents than drugs which lack such specificity.

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LIST OF ABBREVIATIONS

APD	action potential duration
APD_x	action potential duration at x% repolarisation
ΔAPD	percent change in APD at three minutes post-occlusion relative to baseline APD in isolated heart experiments
AS	arrhythmia score; a quantitative measure of arrhythmic events following coronary artery occlusion
D25%	dose producing a response equivalent to a 25% increase from baseline
ED_{50}	dose producing a response equivalent to 50% of maximum; used here for coronary occlusion experiments for effects on arrhythmia score
ERP	effective refractory period
ISI	ischaemia-selectivity index; a quantitative measure of relative potency in ischaemic versus normal tissue
ISI-R	ischaemia-selectivity index for effects on repolarisation
ISI-V	ischaemia-selectivity index for effects on conduction
iT	threshold current for capture in electrical stimulation experiments
MAP	mean arterial pressure
MFF	maximum following frequency
MI	myocardial infarction
r^2	coefficient of determination for a non-linear regression (goodness of fit)
SAR	structure-activity relationships
SCD	sudden cardiac death
SNR	signal-to-noise ratio
V_{max}	maximum rate of rise of a cardiac action potential

ΔV_{max}	percent change in V_{max} at three minutes post-occlusion relative to baseline V_{max} in isolated heart experiments
VT	ventricular tachycardia
VF	ventricular fibrillation
VFt	threshold for electrical induction of ventricular fibrillation

DEDICATION

This thesis is dedicated to the memory of my brother, Adam Vincent Walker.

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First and foremost, I would like to thank my parents. I've spent a good portion of my life at University and I've learnt a few things along the way, one of the most important lessons being that I owe my success more to them and their parenting than to any inherently brilliant insight I might have felt I possessed at one time. My father had me reading by the time I was three, and eagerly fed my insatiable curiosity. He served as an intellectual role model for me, and is an enjoyable companion for discussing all sorts of philosophical and scientific matters. He patiently held his tongue while I went through my "rebellious stage", chanting over and over like a mantra "as long as she stays at univeristy, as long as she stays at university....". My mother was a role model for selflessness, sacrifice, and awareness of the world outside this continent. She told me to reach for the stars, and promised to serve as a rock of love and support throughout my journey. She was true to her word, and I honestly could not have made it without that rock to depend on. My parents taught me that there was nothing I could not do, and fortunately for me, I believed them.

My next debt of gratitude goes to my professor, advisor, and supervisor, Dr. Michael Walker. Despite having to deal with the daily disappointment of leaving a passel of Walkers at home only to encounter yet another one at work, he patiently guided me throughout my many years in the department of Pharmacology. He liked to perpetuate the image of a belligerent, politically incorrect ol' codger, but I often suspected this was a ruse designed to goad students into discovering that if you have an opinion, you should voice it. Nevertheless, I have amassed ample evidence over the years that shows Dr.Walker to be a kind and generous man. He will doubtless deny these accusations, lest rumours of his good nature filter down to the latest crop of undergraduates. I know he will grumble and mutter about this published account of his affection and kind-heartedness, but this thesis would not be complete without acknowledging his role in it. Thank you, Dr.Walker...for everything.

CHAPTER 1: INTRODUCTION

1.1. Acute Myocardial Infarction and Sudden Cardiac Death

Sudden death of cardiac origin is one of the leading causes of death in North America. In the United States approximately 300,000 people die of sudden cardiac death (SCD) each year [1]. Most of these deaths can be attributed to ischaemic heart disease [2], a common manifestation of arterial atherosclerosis. Ischaemic heart disease encompasses two major pathological conditions: angina pectoris and myocardial infarction.

Acute myocardial infarction (MI) is due to sudden occlusion of a coronary artery. Commonly referred to as a "heart attack", acute MI is often a dramatic event accompanied by pain, dizziness, nausea, and syncope, among other symptoms. The pathophysiology of MI relates to the damaging effects of arterial flow blockade on tissue "downstream" from the site of occlusion. This tissue becomes starved of blood and oxygen and is exposed to a buildup of toxic cellular waste products. In this state the tissue is referred to as "ischaemic", and in time the damage becomes irreversible. Death may occur during the initial ischaemic episode or at various stages after the onset of ischaemia due to the anatomical and physiological changes that occur during the process of "infarction" (tissue necrosis and scar formation). The cause of death is usually ventricular fibrillation.

Ventricular fibrillation (VF) is the most severe type of cardiac arrhythmia and the most common cause of death in MI patients. Available interventions for prevention or treatment of VF include antiarrhythmic drug therapy or use of surgically implanted electronic devices. Recent clinical trials such as MADIT [3] suggest that implantable cardiac defibrillators (ICDs) may perform better than currently available antiarrhythmic drugs in reducing mortality in certain patient populations. However, the CABG-Patch trial [4] failed to show a similar superiority in patients with significant heart failure (ejection fraction $< 35\%$). Thus ICD therapy has yet to

replace antiarrhythmic drugs as the treatment of choice for post-MI patients [5;6]. Due to the appreciable value of non-invasive therapy, the search for safer and more effective antiarrhythmic drugs continues to be a worthwhile endeavour.

1.2. Cardiac Arrhythmias

This thesis aims to explore the antiarrhythmic effects of a novel series of compounds against cardiac arrhythmias, particularly those due to myocardial ischaemia. Thus a definition of arrhythmia and brief review of arrhythmogenic mechanisms is presented in this section.

An arrhythmia is an irregular heart rhythm. The mildest arrhythmias are single premature or "extra" beats which occurs even in normal, healthy people from time to time with no ill consequence. The most severe type of arrhythmia is VF which is fatal if left untreated. During VF there is no discernable beating pattern in the heart and thus no effective pumping of the blood. Arrhythmias can also be defined as a *lack* of rhythmic heart beats, as in the case of partial atrioventricular (AV) block, where some signals do not propagate from the atria through to the ventricles. The cause of arrhythmias can often be attributed to structural defects resulting from congenital malformation or disease; however, they may also be induced by cardiac drugs.

The most clinically relevant arrhythmias are tachycardia and fibrillation, especially when they occur in the ventricles. In the atria they are less serious due to the protective nature of AV node delay. It is generally accepted that the AV node serves to protect the ventricles from atrial arrhythmias, as well as coordinating atrial and ventricular contraction. An interesting study suggests that in very large mammals, such as whales and elephants, protection may in fact be the primary function of the AV node [7-9]. Nevertheless, ventricular tachycardia and fibrillation are more serious because of their effect on contraction of the left ventricle, filling time, and effective pumping of blood.

There are three major models of changes in cardiac activity that have been postulated to explain how cardiac arrhythmias are generated. These are: abnormal automaticity, triggered automaticity, and re-entry. Perturbations in automaticity (pacemaker activity) can account for premature beats and transient episodes of tachycardia and have been implicated in torsade de pointes [10], but the primary mechanism of VF in myocardial ischaemia is re-entry.

1.2.1 Re-entrant Circuits

A re-entrant circuit occurs when a cardiac impulse is delayed through a region of tissue and, when emerging from this region of slowed conduction, is able to go back around it and trigger an impulse in tissue which has already recovered from refractoriness [11]. Thus a single impulse can effectively excite the heart more than once by propagating through electrophysiologically heterogenous pathways [12].

Re-entry may be anatomically defined, as in the case of Wolff-Parkinson-White patients, where a fast-conduction pathway provides an anatomical "bypass" from the atria to the ventricles around the normal slow conduction pathway of the AV node [13]. Re-entrant pathways can also be functionally defined, as in acute MI. Ischaemia causes slowing of conduction and increased heterogeneity of refractoriness which can create and sustain sites of unidirectional block that may precipitate re-excitation of surrounding tissue [14].

The course of action for treating re-entrant arrhythmias is to terminate one of the pathways in the circuit. This can be achieved pharmacologically by slowing conduction via inhibition of fast inward (depolarising) sodium currents in ventricular tissue [15]. However, if the degree of conduction slowing is not sufficient, such interventions may actually exacerbate a re-entrant circuit or cause a new one to develop [16]. Another course of action is to prolong refractoriness, so that the impulse coming out of a slow conduction pathway encounters tissue that is unexcitable [17]. This can be achieved by prolonging recovery of sodium channels from

inactivation or by blocking potassium currents responsible for repolarisation, both of which regulate the duration of the refractory period. Theoretically, any intervention which reduces the degree of heterogeneity of the two pathways with regards to conduction or repolarisation times will tend to reduce the incidence of re-entrant circuits. Thus it has been suggested that accelerating conduction in the slow pathway might also be antiarrhythmic [18].

1.3 Antiarrhythmic Drugs

1.3.1 Classification

In 1970, Vaughan Williams [19] established a scheme for classifying antiarrhythmic drugs based on their effects. This was later modified by Harrison [20]. This is still the most commonly used classification system for antiarrhythmic drugs and is shown in Table 1. Numerous attempts have been made to revise and improve this system over the years [21;22]. However, the Vaughan Williams system is simple and useful and remains the clearest and most readily accessible method for categorising antiarrhythmic drugs [23].

The main problem with this system is that few drugs have actions that are restricted to one class. For example, quinidine has class I and, to a lesser extent, class III actions [24]; d,l-sotalol has class II and class III effects [25]; and amiodarone exhibits the effects of all four classes [26]. In addition, antiarrhythmic drugs may have primary and secondary effects which differ in their classifications, as well as active metabolites whose actions may be different from that of the parent drug [27]. Thus each antiarrhythmic drug should be considered a unique pharmacological agent and not interchangeable with other members of its class.

Table 1. Vaughan Williams classification system for antiarrhythmic drugs.

Class	Defined As	Effects	Examples
Ia	Na-channel blocker	Sodium channel block; potassium channel block with intermediate kinetics; increases QRS duration and QT interval of the ECG	quinidine disopyramide procainamide
Ib	Na-channel blocker	Sodium channel block; rapid kinetics; decreases QT interval of the ECG	lidocaine mexilitine tocainide
Ic	Na-channel blocker	Sodium channel block with slow kinetics; increases QRS duration of the ECG	flecainide propafenone moricizine
II	Beta-adrenoceptor blocker	Decrease sinus rate; inhibition of sympathetic effects	propranolol
III	APD prolongation	no effects on conduction; delays repolarization; increases QT interval of the ECG; often potassium channel block and/or slow sodium channel facilitator	bretylium amiodarone sotalol ibutilide
IV	Ca-channel blocker	slows conduction in AV node; decreases heart rate, increases PR interval of the ECG	verapamil diltiazem

Antiarrhythmic drug classification scheme based on the method of Vaughan Williams [12] and Harrison [20]. Classes are shown with category definitions and descriptions of effects on cardiac conduction, repolarisation, sinus rhythm, innervation and the ECG. Examples of conventional antiarrhythmic agents are given for each class and subclass.

1.3.2 Effectiveness of Antiarrhythmic Drug Therapy

Despite the wide variety of drugs available for clinical use against arrhythmias, very few of these have been shown to reduce mortality. Historically, the first series of clinical trials to show a potential benefit in preventing SCD in MI patients involved β-adrenergic receptor blocking drugs. The Beta-Blocker Heart Attack Trial (BHAT) [28] showed that propranolol reduced mortality due to SCD by 28% during a 25-month long follow up period of post-MI patients. In a study of metoprolol, a 36% reduction in mortality was noted by 3 months post-MI [29]. Timolol, another β-blocker, showed a 39% reduction in mortality over a 33 month follow up period post-MI [30]. Many short-term studies have also supported these findings, showing that average reduction in mortality with β-blockers is 34% [29]. Despite these encouraging results, recent meta-analyses indicate that β-blocker therapy has a greater effect on *non*-SCD [31]. Therefore, while β-blocker therapy may be appropriate for patients with congestive heart failure and other cardiomyopathies where non-SCD death is a signficiant risk, it may not be well-suited to MI patients, who are at great risk for SCD [32].

Antiarrhythmic drugs that block sodium channels (Class I) have also undergone extensive clinical testing, but with very disappointing results. The CAST trial of 1989 [33] demonstrated that encainide and flecainide actually increased mortality in post-MI patients and the study had to be terminated prematurely. It was continued as CAST II [34] using moricizine but this trial also had to be terminated early due to an increased risk of fatal arrhythmias. These failures were not limited to the Class Ic drugs: a clinical study of mexilitine (class Ib) was terminated early due to a trend towards higher mortality in the IMPACT study [35]. Lidocaine, another Ib agent, did not significantly reduce mortality in the acute or hospital-monitored phase of MI in randomised, controlled, clinical trials although, interestingly, it did reduce the incidence of VF [36]. Finally, disopyramide (class Ia) was demonstrated to increase mortality over placebo-treated groups [37].

With the negative results of so many Class I drug trials investigators began to focus on prolongation of repolarisation as a preferential mechanism for the prevention of arrhythmias and SCD. Class III drugs all prolong repolarisation, but do so by different mechanisms, which may explain the mixed results of clincial trials with these agents. In the SWORD trial [38] d-sotalol was shown to have no effect on mortality in patients with <30% ejection fractions, but increased risk of death in those with higher ejection fractions. These results are similar to those of the DIAMOND trial of dofetilide [39] which showed that this class III agent does not appear to be associated with excess mortality in patients with an ejection fraction less than 30%. These results suggest that risk/benefit factors (such as the degree of heart failure) may play a role in determining the success of therapy with these drugs to alleviate symptoms of recurrent tachycardias. However, a method for identifying patients who are at an appropriate level of risk for such therapy has yet to be firmly established [40].

Amiodarone, which possesses some class Ib, class II, and class IV actions, aside from its predominant class III effects, was shown to be associated with a decreased risk of mortality in several pilot studies [41]. BASIS [42] showed a decreased risk of death only in patients with ejection fractions >40%, whereas CAMIAT [43] and EMIAT [44] showed a decrease in mortality in those with ejection fractions <40% (which was the criteria for patient enrollment in these two studies). Patients in both CAMIAT and EMIAT who were taking β-blockers along with amiodarone showed the greatest effect of decreased mortality. Despite the success of amiodarone in a variety of clinical trials its use is limited by considerable side effects. In CAMIAT, EMIAT, and the CHF-STAT trials [45], poor patient compliance and significant amiodarone-associated morbidity were noted, as well as potentially lethal pulmonary effects and hepatic and thyroid toxicities. Even when administered in low doses, amiodarone therapy requires long-term surveillance of liver, neurologic, ophthalmologic, thyroid, and pulmonary function, as well as skin condition [46].

The comparable alternative to amiodarone is d,l-sotalol. Unlike d-sotalol, the racemic mixture has significant β-blocking activity. The ESVEM trial [47] compared seven different antiarrhythmic agents and found that d,l-sotalol provided the highest benefit in reducing total mortality and arrhythmia-related mortality when compared to class I drugs. The results of this and a subsequent ESVEM study [48] indicated that while the class III effects of sotalol produced antiarrhythmic effects, the reduction in mortality was due to its β-blocking actions. However, due to the nature of the study it is not known whether d,l-sotalol was truly beneficial for these patients, or is just preferable to class I therapy. Sotalol is not associated with low patient compliance like amiodarone, and it lowers defibrillation thresholds which could be useful for patients with ICDs [46]. However, amiodarone slows the cycle length of arrhythmias to a greater extent than sotalol, which could result in decreased need for device intervention.

To summarise the above, despite the wide range of antiarrhythmic drugs available for clinical use, very few have been shown to be clinically effective in reducing mortality and morbidity in post-MI patients. Consequently, this thesis seeks to explore new mechanisms of antiarrhythmic drug action in a series of novel antiarrhythmic agents in the hopes of improving the effectiveness of antiarrhythmic drug therapy.

1.3.3 Strategies for Antiarrhythmic Drug Development

Based on the disappointing results of so many clinical trials over the years, investigators are attempting to redefine their approach to antiarrhythmic drug development. Certainly there are many clinical considerations that might address the issue of unsatisfactory drug trial results including enrollment criteria, protocols and experimental design, monitoring of appropriate endpoints, and consideration of patient risk profiles. In a recent review, Kowey [26] argued for a fuller appreciation of the pharmacokinetics of antiarrhythmic drugs and consideration of patient profiles with respect to disease state, concurrent drug therapies and endogenous or age-related

metabolic variations. However, development of new and better antiarrhythmic agents will almost certainly play a significant role in the effort to improve patient survival.

Rosen [49] suggested that the problem with past approaches to drug development was to presume that a potentially lethal arrhythmia results from a mechanism so unapproachable by specific targeted therapy that an array of targets is best sought. This empirical approach results in current treatments often being unrelated to considerations of underlying causes or mechanisms associated with specific arrhythmias. Thus, many antiarrhythmic agents lack specificity either for the type of arrhythmia being treated or the pathology causing the arrhythmia.

In 1991 the Sicilian Gambit investigators [23] introduced the concept of the "vulnerable parameter", defined as "the electrophysiologic determinant of an arrhythmia that is most susceptible to pharmacologic (or other) alteration, while manifesting a minimum of undesireable effects when manipulated". Clearly a targeted approach to drug development is more desirable than a purely empiric approach, but at present there are few arrhythmogenic mechanisms about which we know enough to direct the course of therapy with the required specificity. Pathology targeting is one approach to the concept of the vulnerable parameter, whereby the source of arrhythmogenic stimuli is pharmacologically altered while unaffected tissue retains normal electrophysiologic function [50]. This was the rationale behind the central hypothesis of this study, which is that drugs which preferentially act in ischaemic tissue provide superior protection against ischaemia-induced arrhythmias. In this case, the pathological target is ischaemia because arrhythmias are dependent upon disordered electrogenesis in the ischaemic zone of the heart during acute MI.

1.3.3.1 Ischaemia-Induced Arrhythmias

Ischaemia is a highly arrhythmogenic condition [51]. Experimental observations shows that it is difficult to provoke ventricular tachycardia (VT) or VF in normal tissue using premature

stimuli, but in ischaemic tissue premature beats readily lead to tachyarrhythmias [52]. Han and Moe [53] observed that premature ventricular beats during ischaemia led to rapid induction of ventricular fibrillation and further, that they exacerbated dispersion of refractoriness, a situation believed to be arrhythmogenic [54]. Clinically, ambulatory monitoring of patients who eventually succumbed to sudden cardiac death showed that incidences of fatal VF were invariably preceded by an extrasystolic beat [55-57].

A mechanism for induction of VF by premature beats was recently suggested by Pastore et al [58] who showed that application of a premature stimulus in electrically uncoupled tissue can provoke an arrhythmogenic form of electrical alternans (beat-to-beat changes in action potential morphology) where gradients of repolarisation become more severe and re-entrant VF can occur. Ischaemia causes cells to uncouple via effects on gap junctions [59], therefore this might account for arrhythmogenesis in ischaemic tissue. While the precise mechanism of ischaemia-induced arrhythmias is not known, the fact remains that ischaemic tissue is much more susceptible to arrhythmogenic stimuli than electrophysiologically normal tissue and this provides a rationale for the development of ischaemia-selective antiarrhythmic drugs.

1.3.3.2 Electrophysiologic Effects of Ischaemia

Before discussing ischaemia-selective antiarrhythmic drugs a brief review of the effects of ischaemia on the cellular ionic environment is warranted. The arrhythmogenic effects of ischaemia are most likely a function of changes in cellular electrophysiology that occur during ischaemia and a large body of literature exists regarding the effects of ischaemia on cell physiology and function (see Gettes [60] and Cascio et al [61] for reviews). Acute ischaemia results in depolarization of the resting membrane potential, decreased V_{max} and amplitude of the action potential, and decreased action potential duration [62-64], as well as increased resistance of intercellular coupling [65-67]. A complex variety of metabolic and physiologic alterations in

the cell and its environment act and interact in various ways to produce these ischaemic effects. This discussion will focus on the major effects of ischaemia, particularly those that represent potential targets for antiarrhythmic drug action.

1.3.3.2.1 Intracellular Sodium

The transmembrane sodium gradient is the driving force behind the voltage-gated sodium current, the Na^+/H^+ exchanger, and the Na^+/Ca^{2+} exchanger. It also affects the Na^+/K^+/ATPase pump, the Na^+/HCO_3^- co-transporter, the $Na^+/K^+/2Cl^-$ co-transporter, and the sodium-activated potassium current, $I_{K,Na}$. Therefore, changes in intracellular sodium will affect a variety of membrane transport mechanisms and ionic currents, leading to alterations in cellular electrophysiology.

Intracellular sodium concentration ($[Na^+]_i$) rapidly increases during ischaemia [61]. One mechanism for this could be depressed efflux of sodium via inhibition of the Na^+/K^+/ATPase pump, however rising $[Na^+]_i$ would be expected to stimulate this process rather than inhibit it. Another mechanism could be increased activity of the Na^+/H^+ exchanger due to acidosis, however Pike et al [68] did not detect significant alterations in Na^+/H^+ exchange in their studies of ischaemic rat hearts. Maddaford and Pierce [69] have shown an important role for Na^+/H^+ exchange during reperfusion arrhythmias but they noted that during ischaemia the exchanger is less active. Xiao and Allen [70] showed that block of the Na^+/H^+ exchanger did not prevent a rise in $[Na^+]_i$ during ischaemia, but it did reduce the rise in $[Na^+]_i$ observed during reperfusion. On the other hand, block of the voltage-gated sodium current abolished the ischaemia-induced rise in $[Na^+]_i$ but had no effect on $[Na^+]_i$ during reperfusion. This study and others [71] point to the importance of the voltage-gated sodium current as a major contributor to rising $[Na^+]_i$ during ischaemia.

1.3.3.2.2 Extracellular Potassium

Accumulation of extracellular potassium is another important consequence of ischaemia. This leads to depolarisation of the resting membrane potential and an incease in the threshold required for triggering an action potential [72]. Initially, the rise in membrane potential from negative values towards zero is more rapid than the increase in threshold, resulting in a transient increase in cell excitability [73]. The rapid increase in extracellular potassium concentration ($[K^+]_e$) is observed as early as 15 seconds after the onset of ischaemia [74]. Whalley et al [75] reported a voltage-independent effect of elevated $[K^+]_e$ on membrane potential that further depressed V_{max} of the action potential during ischaemia. Another consequence of high $[K^+]_e$ is post-repolarization refractoriness [73]. On its own, high postassium concentration shortens action potential duration (and thus the refractory period) [76]; however, its effects on membrane potential delay the recovery from inactivation of sodium channels and prolong the effective refractory period (ERP) past the point of repolarisation [77].

The rise in $[K^+]_e$ in the early stages of ischaemia may be due to a number of factors, as reviewed by Cascio et al [78] and Wilde and Aksnes [79]. Depletion of ATP, intracellular and extracellular acidification, activation of outward K^+ currents such as the ATP-sensitive channel (K_{ATP}), the arachidonic acid sensitive channel (K_{AA}), and the sodium-activated channel ($I_{K,Na}$) may all be contributing factors. The well-documented increase in intracellular sodium and calcium may be responsible for some of the passive outflow of K^+. It has also been suggested that the passive flow of anions such as chloride [80-82] or inorganic phosphate [83] might contribute to this as well.

A plateau phase of potassium depletion occurs after approximately 10-15 minutes of ischaemia [84]. This may be due to activation of the Na^+-K^+-ATPase pump leading to active uptake of potassium through this channel, or passive leaking of potassium out of the ischaemic zone [85]. A third phase of extracellular K^+ accumulation occurs after the plateau phase and

probably represents compensation for the marked increase in intracellular sodium and calcium and failure of the Na^+-K^+-ATPase pump [61].

1.3.3.2.3 Changes in pH

Another key feature of ischaemia is decreased extra- and intracellular pH (pH_e and pH_i, respectively) [86]. One of the most important contributors to this acidosis is the Na^+/H^+ exchange pump. Under normal conditions it maintains pH_i in myocardial cells at an alkaline 7.3. It is believed that this pump, which takes in Na^+ in exchange for extruding H^+, is closely linked to the Na^+/Ca^{2+} pump (and thus contractility), which extrudes Ca^{2+} in exchange for Na^{2+} [87]. The Na^+/H^+ pump has been implicated as a major factor in reperfusion injury, as reviewed by Karmazyn and Moffat [88].

During ischaemia excess protons are generated by a variety of processes such as anaerobic metabolism, which leads to an increase in lactate and accumulation of CO_2 [89]. These excess protons are removed to the extracellular space via the Na^+/H^+ pump, where lack of efficient buffering capacity rapidly leads to acidosis. It has been shown in some studies that after the onset of ischaemia the buffering capacity within the cell is quickly overcome and pH_i begins to decline shortly after pH_e [61]. Studies in isolated blood-perfused rabbit hearts showed that pH_e falls continuously for 30 to 40 minutes, reaching levels of 6.0, after which time the decrease diminishes [67]. This plateau effect is likely due to inhibition of anaerobic glycolysis. In isolated Langendorff-perfused ferret hearts [90] investigators found that pH_i continues to decrease even after the decrease in pH_e levels off, and falls to at least 5.5.

Acidosis has been shown to produce a small depolarisation of the resting membrane potential [91]. Protons also inhibit voltage-gated sodium [92] and calcium [93] currents in a concentration-dependent manner. The result of this is decreased action potential amplitude and V_{max}, and negative inotropy. Negative inotropy is also due to other factors such as inhibition of

calcium uptake and storage by the sarcoplasmic reticulum, and inhibition of calcium binding to troponin C of the contractile apparatus [94]. Intracellular acidosis also decreases gap junction conductance and likely contributes in this way to cell-cell electrical uncoupling.

1.3.3.3 Ischaemia-Selective Antiarrhythmic Drugs

If ischaemic tissue is responsible for arrhythmogenesis, and if disturbing conduction or repolarisation in normal tissue is potentially proarrhythmic, then drugs which are preferentially active in ischaemic conditions should make more effective antiarrhythmic agents than drugs which have significant effects in normal tissue. Recent evidence for this hypothesis comes from several studies.

Bui-Xuan et al [95] demonstrated that flecainide increased ventricular fibrillation threshold (VFt) in the absence of ischaemia, but this effect was abolished during ischaemia. Barrett [96] also showed that flecainide lacks ischaemia selectivity and was less effective at suppressing ischaemia-induced arrhythmias than drugs which were more potent under ischaemic conditions. Aupetit et al [97] showed that flecainide, lidocaine, and disopyramide increased ventricular fibrillation threshold in the absence of ischaemia, but failed to prevent the ischaemia-induced decrease in ventricular fibrillation threshold (VFt), and in fact accelerated the decline. Yang and Roden [98] and Duff et al [99] found that dofetilide was less effective under conditions of elevated extracellular potassium concentration (one of the consequences of ischaemia [72]). Thus dofetilide selectively prolongs action potential duration in normal tissue, with limited effects in ischaemic tissue.

On the other hand, Bui-Xuan et al [95] showed that verapamil produced little effect on VFt prior to ischaemia, while preventing or considerably delaying the ischaemia-induced fall in VFt following occlusion. This supports similar findings for verapamil by Curtis et al [100] who first suggested that targeting drugs to ischaemic tissue may be beneficial. Interestingly, Farkas et

al [101] have recently reported that the calcium channel blocker mibefradil has limited antiarrhythmic efficacy due to inadequate ischaemia-selectivity, even though, like verapamil, its primary effects in cardiac tissue are mediated by blockade of voltage-activiated L-type calcium channels. This suggests that ischaemia-selectivity is not necessarily a function of the channel to which a drug binds.

The mechanism by which some antiarrhythmic drugs show selectivity for ischaemic tissue is due to their preference for specific channel states that predominate in the depolarised membrane potential environment of ischaemia [102], specifically the inactivated state of the sodium channel [103]. However, this property also confers a certain degree of rate-dependence since the frequency of channel activation will dictate availability of the preferred state in the same manner as membrane voltage. This means that under conditions of rapid heart rates the action of such drugs will be potentiated. High heart rates may therefore serve to confound differences in drug effect between normal and ischaemic tissue and this may be responsible for the paradoxical proarrhythmic tendencies of many antiarrhythmic drugs [104].

Evidence for this comes from Ye et al [105] who found that frequency-dependent effects in normal tissue underlied the proarrhythmic tendencies of propafenone. Carson et al [106] suggested that the pro-fibrillatory tendencies of lidocaine in regionally ischaemic pig hearts were due to its conduction-slowing effects in normal tissue during tachycardia, while already providing a significant level of conduction suppression in the ischaemic zone. Campbell et al [107] found that while lidocaine and amiodarone were relatively selective for ischaemic tissue, their rate-dependence was more pronounced in normal tissue, leading to decreased effects in ischaemic tissue at rapid heart rates. Havercamp et al [108] and Fazekas et al [15] agreed that the rate-dependent actions of lidocaine on normal tissue, when combined with ischaemia-dependent slowing of conduction, would create heterogeneities in conduction velocity that could set up re-entrant circuits. Starmer et al [109] reported that simulations using hypothetical rate-dependent

and non-rate dependent sodium channel blocking drugs showed the former had increased proarrhythmic effects due to prolongation of the duration of the "vulnerable window" for development of reentrant circuits. Janse wrote "suppressing conduction in normal tissue may actually create re-entrant circuits" [110]. This might explain the failure of frequency-dependent antiarrhythmic drugs to yield successful candidates for therapeutic agents.

The above suggests that if sufficient degrees of ischaemia-selectivity are necessary for superior antiarrhythmic protection, this property must be obtained in the absence of equivalent effects in electrophysiologically normal tissue. Consequently, rate-dependence may not be an appropriate mechanism for conferring ischaemia-selectivity. Thus it has been suggested that taking advantage of the altered environmental conditions in myocardial ischaemia might prove a better approach than targeting specific ischaemia-induced channel states. This principle has previously been demonstrated in this laboratory for series of novel antiarrhythmic agents [50;110-112] and standard antiarrhythmic drugs [103;111;113].

1.3.3.4 Ischaemic Targets for Antiarrhythmic Drugs

A logical target for antiarrhythmic therapy might be the factors responsible for creating arrhythmogenic conditions during ischaemia. However, a review by Curtis et al [111] noted that investigators have implicated at least 15 possible substances (ions, proteins, and other molecules) which are present during ischaemia and may have arrhythmogenic effects. The task of targeting antiarrhythmic therapy to several substances at once is formidable, even considering the possibility that a smaller fraction of them may be responsible for the majority of arrhythmic events occurring during ischaemia. Targeting electrophysiologic variables in ischaemic tissue may therefore be more practical than trying to ameliorate the process of ischaemia itself. With that said, however, Baker and Curtis [112] have recently demonstrated that the platelet-activating factor antagonist, BN-50739, can suppress ischaemia-induced VF when applied to the ischaemic

zone of a regionally-occluded isolated rat heart using a specially designed dual-lumen cannula; but when applied selectively to the perfused region of the preparation BN-50739 failed to suppress VF.

There are other means by which drugs may be targeted to ischaemic tissue. During ischaemia, affected cells swell in volume. This could provide a target for drug potentiation, as suggested by Wright and Rees [113]. Certain drugs have been shown to be less effective under conditions of cell swelling and the authors postulated that this could be implicative in pro-arrhythmic, or at least insufficient, antiarrhythmic drug action. They suggested that understanding the process of ischaemia-induced cell-swelling could lead to the design of drugs whose actions are potentiated in swollen (i.e. ischaemic) cells.

The acidic environment of ischaemic tissue may also provide a means of targeting drug action to ischaemic tissue. Acidosis can depolarise membrane potential and this may potentiate the actions of certain antiarrhythmic drugs [114]. Drug molecules could be designed in such a way as to be affected directly by high proton concentrations [50]. Specifically, compounds with appropriate pK_a values, where the more charged form is favoured by acidic conditions (i.e. $pK_a \sim 6.4$), would be expected to show greater potency in ischaemic tissue if the charged form was the active form. Such compounds have been developed [115] and Yong [116] recently showed that such drugs were more effective antiarrhythmic agents than those with high pK_a values (~ 7.4).

1.4 Thesis Rationale

The goal of this laboratory is the elucidation of a mechanism for antiarrhythmic protection in the setting of myocardial ischaemia. In 1984, Curtis et al [100] suggested that calcium channel blockers selective for ischaemic tissue might be better antiarrhythmics for ischaemia-induced arrhythmias. This hypothesis was developed further with respect to sodium channel blocking drugs that might act selectively in cardiac tissue to block channels externally

and with increased potency in ischaemic tissue [117;118]. Studies with certain kappa-opioid drugs that possessed antiarrhythmic activity unrelated to their effects on opiate receptors [119-122] led to development of a lead compound that appeared to meet the criteria previously defined. This compound, RSD921, appeared to act at an external cellular site and had a chemical structure that could be manipulated to affect pK_a. By varying the concentration of charged species at normal and acidic pH it was expected that potency could be preferentially retained in ischaemic tissue.

In the mid-1990's, structure-activity relationship studies led to a synthesis programme that yielded compounds with the desired pK_a values. These compounds were shown to alter indices of electrophysiologic activity (reflected in ECG changes) in a way which was selective for conditions of simulated ischaemia (high $[K^+]$ and low pH) [50;123;124]. In these studies, such compounds were associated with improved antiarrhythmic efficacy against ischaemia-induced arrhythmias. The degree of specificity for ischaemic-like conditions was in some instances several orders of magnitude greater than that of current antiarrhythmic drugs [50;115;124]. Recently, Barrett et al [125] reported on the effects of one such compound, RSD1019, in anaesthetised rabbits subjected to regional ischaemia. Compared to lidocaine and tedisamil, RSD1019 showed greater potency in ischaemic tissue for prolonging monophasic action potential duration and was a more effective antiarrhythmic agent.

This study is a continuation of the above work and seeks to demonstrate a significant relationship between electrophysiologic actions that are selective for conditions of physiological ischaemia and effective suppression of ischaemia-induced arrhythmias, using a series of related compounds in the same species.

1.5 Experimental Outline and Procedures

Six structurally-related test compounds were selected from an SAR (structure-activity relationship) series to test the above hypothesis. Attempts were made to relate the actions of these drugs in normal and ischaemic tissue to their antiarrhythmic actions. The experimental outline is presented below in numbered format and relevant background information is included where it pertains to the selection and application of specific methods used herein.

1) Antiarrhythmic activity against ischaemia-induced arrhythmias was evaluated using acute regional coronary artery occlusion in anaesthetised rats. The end-point was occurrence of arrhythmias summarised as an arrhythmia score (AS). The latter is a normally-distributed, quantitative representation of arrhythmic events following coronary occlusion which was fit to a sigmoidal dose-response equation using non-linear regression.

2) Electrical stimulation of the left ventricle was performed in anaesthetised rats to evaluate the antiarrhythmic effects of test compounds against non-ischaemic arrhythmias. Specifically, the potency for elevating threshold current required to induce ventricular fibrillation was estimated and compared with potencies for drug effects on excitability and repolarisation as measured from the electrocardiogram and electrical stimulation variables. Potency indices were calculated based on dose-response curves fit using non-linear regression.

3) Drug effects in normal and ischaemic tissue were evaluated by inducing regional ischaemia in isolated rat heart Langendorff preparations via occlusion of a branch of the left coronary artery. Cardiac action potentials were recorded from normal and ischaemic regions simultaneously using optical detection techniques with a voltage-sensitive dye. The relative

effects of drug on excitability and repolarisation in normal versus ischaemic myocardium were determined by calculation of an "ischaemic-selectivity index" using one of three formulae.

4) The final component of this study involved correlation analyses between indices of antiarrhythmic activity and ischaemia-selectivity to explore the hypothesis that ischaemia-selectivity provides a mechanism of antiarrhythmic protection in the setting of acute myocardial infarction.

1.5.1 Dose-Response Curve Analysis

Dose response curve analysis was used to determine various indices of antiarrhythmic protection. In ischaemia-induced arrhythmia experiments dose-response curves were fit to a predefined equation using non-linear regression in order to estimate potency, slope, and goodness of fit. In electrically-induced arrhythmia experiments dose-response curves were fit so that estimates of potency could be determined for antiarrhythmic protection, electrical stimulation variables, and ECG changes.

The nature of the dose-response relationship relates pharmacological responses to the administration of a specific drug (i.e. assumes causation) [126]. In pharmacology, particularly clinical studies, evidence of a dose-response relationship is taken to be a more compelling finding than evidence of a positive effect that does not appear to be dose-related [127]. The first objective of any dose response study is to determine whether there was, in fact, a drug effect. When this can be demonstrated it is often desirable to characterize the nature of this relationship, preferably in mathematical form.

Analytical strategies for exploring dose-response relationships can be divided into two basic approaches according to Ruberg [128]. The first is analysis of variance (ANOVA) followed by the use of multiple comparison tests or their equivalents. ANOVA simply tests whether or not

a group of means come from the same population; each dose level is treated as a discrete group of data. If the null hypothesis is rejected it does not provide details as to which means are from the same population and which are different, therefore the next step is usually a multiple comparison test such as the Tukey test or Newman-Keuls test [129]. Multiple comparison tests and contrast tests evaluate the existence of a drug effect, but they do not provide any information about the specific mathematical nature of the dose response relationship.

An alternative approach to analysing dose response studies is the application of regression analysis. Regression is useful in cases where patterns are likely, but not certain, and it offers an approach that is more trustworthy than simply assuming a particular relationship. When appropriate, it is certainly more powerful than any individual comparison approach [129]. Regression analysis requires that one specify the nature of the relationship in mathematical form, or at least provide a variety of such functions from which the best fit may be determined. Linear models of the simple form "$y = mx + b$" are typically involved, but in biological systems it is common to use more complex, non-linear models. Non-linear regression was used in this thesis.

1.5.1.1 Non-Linear Regression

Selecting an appropriate model for regression analysis can be difficult. To describe the nature of a dose-response relationship, for example, one must possess a certain degree of knowledge regarding the underlying biological processes involved. There may be several mathematical functions that appear to describe the data equally well, yet differ substantially in their estimated properties. Functions should therefore be selected based upon a hypothetical physical or molecular model whenever possible [130]. The selection should not be based entirely on statistical information (such as goodness of fit), but on the physical plausibility of the model described by the function and consistency with other data. Chuang-Stein and Agresti [131] suggest that a well-fitting model should describe the nature of the association, provide

parameters for describing the strength of the relationship, provide predicted probabilities for the response categories at any dose, and help determine the optimal dose.

Many dose-response relationships in biology can be modelled by a logistic function of the form:

$$y = \frac{x^n}{1 + x^n}$$

{Equation 1}

A more familiar form of this equation, and the one most often seen in pharmacology, is described by:

$$y_e = \frac{k_1 x}{k_2 + x}$$

{Equation 2}

where y_e = response, x = dose or concentration, k_1 corresponds to the maximum possible response (y_{max}) and k_2 represents the ED_{50} (value of x for half-maximal response) [132]. This equation represents a rectangular hyperbola and has the same form as the Michaelis-Menten equation which describes the rapid equilibrium kinetics of enzyme systems.

Non-linear regression techniques assume that the variance associated with y is not dependent on any value of y or x [133]. Thus there is an error term associated with equation 2 such that $y_o = y_e + e_i$ where y_o is the observed value of y and e_i is the experimental error associated with each y. The assumption of equal variance of errors in non-linear regression takes into account the measurement errors inherent in the apparatus, but not necessarily human errors [134]. Thus, for example, the error value e_i is associated with the precision of a pipette but does not take into account an experimenter accidentally including an air bubble in the pipette. It is also assumed in non-linear regression that the distribution of all errors is linear. This is not always the case, however. For example, errors associated with scintillation counts in radioligand

studies are distributed in a Poisson fashion [135]. Furthermore, regression assumes homoscedasticity, that is equal variances of y for different x populations. Transformation of normally-distributed data, such as taking the log of the dose to linearise the dose response graph, may result in heteroscedasticity. A log transformation of y may correct this.

Both linear and non-linear regression "best fit" procedures seek values for estimates which reduce the "sum of squares" value (sum of the squares of the distances of the data points to the curve) [133]. This is called the "least squares method" and is appropriate when the above assumptions about error are met. Such analyses are performed by computers using starting values for each parameter to be estimated. These values can be estimated by the operator using an "educated guess". It is generally not difficult to provide reasonable estimates assuming one has a good understanding of the principles underlying the biological system being studied, and most analysis programs are robust for departures from the true value. The computer uses an iterative procedure, changing the estimates slightly each time and determining the effect on the sum of squares value.

With regression analysis computation the equation and the parameters which provided the lowest sum of squares value in the analysis form the results. The question of how well the data fits this equation (and the curve described by it) is determined by "goodness of fit" measurements. There are a variety of statistical tests one may perform to measure goodness of fit and this may aid in determining what equation best describes the process under study, but visual inspection of the graphical function is necessary before drawing conclusions about relationships [128]. What may be, statistically, the "curve of best fit" may be meaningless in the context of what is known about the biological system. For example, one may be seeking a function to describe the blood-pressure lowering effects of a certain drug. One may describe the effects on blood pressure as a percent decrease from control. Logic would dictate that a result greater than 100% is not possible, since one cannot have a blood pressure which is less than zero. However,

the computer may describe a function whose y-maximum is 200%. This function may have a higher goodness of fit estimate than a function whose maximum is 100%, but we know the former to be impossible.

If visual examination of the data does not initially rule out the possibility of the results being a fair description of the system, one may proceed to statistical tests of goodness of fit. The computation provides a value for r^2, the coefficient of determination. This value is between one and zero and describes the fraction of the overall variance of the y values that is reduced, or accounted for, by fitting the data to the curve. An r^2 value of 1 represents a perfect fit. Another way of examining goodness-of-fit is to make a plot of the residuals versus x. The residuals are the distances of the y points from the fitted curve. If the equation is appropriate, one would expect the residuals to represent only experimental error and therefore be randomly distributed (with respect to x) with both positive and negative values. If the equation is inappropriate residuals may cluster about certain values of x. Such a result indicates a systematic deviation from the curve.

The parameters in the regression equation generally represent values of specific interest. For example, in equation 2 above one may determine the y_{max} and the ED_{50} of the dose response curve by estimating the values k_1 and k_2. Most analysis programs also report an error associated with the estimated value, however certain considerations must be included. In non-linear functions errors are neither additive nor symmetrical and exact confidence limits cannot be calculated [129]. The reported standard error values for parameter estimates are based on linearising assumptions and will always underestimate the true uncertainty of any nonlinear equation. One should not rely on standard error estimates when comparing two models, for example [130]. They are quite useful, however, as an informal measure of goodness of fit. Since they are underestimates one can reject values with high standard errors without hesitancy. Large

error values may indicate that the model is inappropriate, that there is unacceptable scatter of data, or that too small a range of x populations were sampled [128].

In conclusion, to use non-linear regression effectively, an intuitive feel for the selected equation and the physical model it represents is important. Results from regression analysis should be visually examined and subjected to critical thought. Statistically meaningful numbers may be valueless in the context of the "real" biological world and the computation does not make such a distinction. For this thesis non-linear regression analysis was used in coronary occlusion and electrical stimulation experiments.

1.5.2 Optically Recorded Action Potentials

A large portion of the work of this thesis constituted the development of a method that would allow for quantification of the degree of selectivity of electrophysiologic drug effects in normal versus ischaemic tissue. This will be discussed in the following section along with background information about the optical recording technique.

1.5.2.1 Development of a method for measuring ischaemia-selectivity

Originally our laboratory investigated the actions of compounds similar to those used in this thesis under conditions of simulated ischaemia. Potassium concentration and pH levels were adjusted in a PIPES-buffered perfusate to be equivalent to those found under conditions of ischaemia (12 mM potassium and pH 6.4, respectively [61]). While these represent two of the major changes that accompany ischaemia, they were not a true representation of physiological conditions following occlusion of an artery and were, by necessity, global in their effect on the heart. There was no hypoxia, and since perfusion was continuous, no lack of washout. Morena et al [136] demonstrated that the effects of physiological ischaemia may differ from those of

hyperkalemia and acidosis alone. Nevertheless, the method proved sufficient as a rapid and effective means of screening compounds for potential ischaemia-selectivity.

In this early technique, electrophysiologic effects were measured using the ECG, and a ventricular balloon monitored left ventricular pressure continuously during the cardiac cycle. Specifically, the effects of drug on heart rate (RR Interval), PR Interval, QRS Duration, maximum systolic pressure, end diastolic pressure, and rate of change of pressure (dP/dt) were measured from polygraph recordings. Because the electrocardiogram of an isolated rat heart does not show a clear T-wave, measurements of repolarisation effects could not be made. However, since the majority of drugs being tested were strong sodium channel blockers this was not a major limitation. Nevertheless, the ECG is a marker of global electrophysiologic activity which does not allow for studies of regional effects.

In past experiments ischaemia-selectivity ratios were calculated from dose-response curves for normal and simulated ischaemia experiments. The D25% (dose producing a 25% change from control) for effects on PR Interval were determined by fitting dose-response curves by hand, and by extrapolation. Slope was assumed to be equal for both curves. This method was most effective when drug effects on PR Interval were significant, and when such effects varied significantly between normal and simulated ischaemia conditions (ie. large degree of separation between curves). However, because QT Interval cannot be determined in isolated rat hearts no estimates of ischaemia-selectivity with regards to repolarisation effects could be made.

In this thesis, modification of the above method involved three objectives: 1) to reproduce as accurately as possible the conditions of regional ischaemia found physiologically upon occlusion of a coronary artery, 2) to measure drug effects on both conduction and repolarisation in a more specific manner, and 3) to determine a quantitative index of ischaemia-selectivity that allowed for significant comparisons between closely-related compounds.

To address objective #1 no-flow ischaemia was used rather than a modified buffer. To address objective #2 a stimulation protocol was used similar to that of the electrical stimulation experiments performed in this thesis. Measurements such as iT (threshold current for capture), tT (threshold pulse width for capture), ERP (effective refractory period), and MFF (maximum following frequency) provided more specific information about the electrophysiologic effects of test compounds on both conduction and repolarisation that could be reasonably extrapolated to effects on specific ionic currents. However, such a protocol, which uses the ECG as an endpoint, required a global regimen of ischaemia be used rather than regional. The ventricular balloon was dispensed with since the effects of global ischaemia were severe enough that contraction fell to very low levels within the first minute of ischaemia and none of the drugs tested showed any significant effects on contraction.

Pilot experiments revealed several difficulties with such a protocol. The effects of global ischaemia were severe, and it became difficult to capture and pace hearts in order to perform the stimulation protocols. It necessitated beginning the protocols at very early times after commencement of global ischaemia, when effects were not significant enough to produce results that differed statistically from control data. This made estimation of an ischaemia-selectivity index difficult. Furthermore, ventricular fibrillation occurred often during stimulation protocols since ischaemia is a very arrhythmogenic condition. In these cases VF was not readily reversible, despite attempts at defibrillation. Where hearts could be defibrillated this produced subsequent effects on the electrophysiologic parameters being measured, and resulted in large variability between hearts. Thus, early experiments failed to meet the objectives outlined above.

The next step was to incorporate direct measurements of drug effects on the cardiac action potential. At first, monophasic action potential recording techniques were used. Electrodes were fashioned in the labortory using small silver-chloride tipped wire electrodes. This method allowed for the use of regional ischaemia which satisfied objective #1 above. Furthermore, the

small size of the electrodes allowed for localised recording from multiple sites. Specifically, recordings could be made from normally-perfused and ischaemic regions simultaneously. It was anticipated that this would reduce variability and thus better address objective #3.

Monophasic action potential electrodes require that pressure be applied to the preparation in order to depolarise the tissue beneath the electrode head and thus provide a reference [137]. This pressure has to be maintained at a constant level while at the same time allowing for the motion of a beating heart. Spring-loaded electrode housings were constructed and pilot experiments were performed at various tensions and levels of applied pressure. Unfortunately, the amount of pressure required for clear signals resulted in tissue damage over the course of the experiment. Tissue beneath the head of the electrode began to show signs of ischaemia and physical damage during the time course of the experiment and this coincided with action potentials that were steadily decreasing in amplitude and showing changes in morphology. This may have been significant because of the small size of rat hearts relative to larger species in which monophasic action potentials have been recorded successfully over longer periods of time [138;139]. The major concern, however, was that monophasic action potential electrodes may be picking up electrotonic influences from other parts of the heart (ie. remote activity), which again would be expected to show more prominance in recordings from small hearts. This perhaps contributed to the lack of significant differences observed between the two recording areas in pilot experiments. After months of modifications without success, an alternative method was discovered that addressed the problems.

Optical recording of action potentials fulfilled all of the above criteria. It allowed for regional ischaemia, simultaneous recording from perfused and non-perfused zones of tissue, and it was non-invasive so that no tissue damage occurred. The size of the area from which signals were recorded could be controlled by restricting the area of illumination, with the upper limits being determined by the dimensions of the photodiode. There is no remote activity since signals

are optical in nature. Depolarisation and repolarisation times could be accurately determined from the high quality signals obtained with the optical system. With these considerations, variability was reduced to the point where significant differences in electrophysiologic function between perfused and ischaemic zones could be measured in both drug-treated and control hearts. Thus the above objectives were satisfied: regional physiological ischaemia could be used, drug effects on conduction and repolarisation could be measured simultaneously, and data allowed for determination of ischaemia-selectivity using a quantitative index of effects in normal and ischaemic tissue.

The use of optically recorded action potentials in cardiac research is relatively recent and, while growing in popularity, is still limited to a few laboratories in North America. The equipment used for these experiments was designed and constructed in the laboratory of Dr. Guy Salama in Pittsburgh, Pennsylvania and training on the use of optical detection systems was received in the laboratory of Dr. David Rosenbaum in Cleveland, Ohio. The following sections give some background information about the history and development of optical action potential recording techniques using voltage-sensitive dyes.

1.5.2.2 Voltage Sensitive Dyes

The use of voltage-sensitive dyes as probes for membrane potential began in the 1960's in the field of neuroscience. Cohen et al [140] and Tasaki et al [141] reported on changes in intrinsic light scattering, birefringence, and extrinsic dye fluorescence associated with squid giant axon neuronal action potentials. The initial idea was to observe optical changes as a possible means of elucidating the molecular mechanisms of nerve excitation. However, it soon became apparent that fluorescence changes depended on transmembrane potential and that optical methods might therefore be used to monitor membrane potential in cells that were not accessible to microelectrodes [142]. At that time, available dyes yielded small signals and were

not suitable for tissue with fast-responses. However, meticulous studies of hundreds of novel compounds eventually yielded dyes with significantly faster response times [143].

Voltage-sensitive dyes fluoresce when excited by light of a specific wavelength. The difference in wavelength between emitted fluorescence and excitation (i.e. Stoke's shift) is typically on the order of 100 nm, making it possible to use optical filtering to selectively record dye fluorescence without contamination by the excitation light. The change in fluorescence in response to changes in local membrane potential is linear, therefore these dyes accurately depict the relative change in transmembrane potential and their signals closely resemble action potentials recorded with conventional microelectrode techniques [144;145]. The magnitude of the voltage-related absorbance change depends on the wavelength of the incident light and each voltage-sensitive dye has its own spectrum. The magnitude of this change generally represents about a 5% to 10% change from baseline levels, thus signals are typically amplified. With some dyes, the action spectra between cardiac muscle and nerve tissue differ, which makes them particularly useful for monitoring cardiac action potentials without contamination from neuronal impulses.

The aminonaphthylethenylpyridinium (ANEP) family of dyes was developed by Loew and colleagues [146]. The submillisecond response times of these dyes make them particularly suitable for cardiac action potential studies. ANEP dyes are essentially non-fluorescent in water and become strongly fluorescent upon binding to membranes, therefore contamination from unbound dye molecules does not occur to any significant degree. The dye "di-4-anepps", used in this thesis, exhibits a fairly uniform 10% decrease in fluorescence intensity per 100 mV increase in voltage potential, therefore the fluorescence signal represents an inverted action potential. The maximum absorbance and emission wavelengths of di-4-anepps is 497 nm and 715 nm, respectively.

Voltage sensitive dyes are available that produce no detectable changes in maximum upstroke velocity, duration, or amplitude of cellular action potentials [147], but phototoxic damage may occur when dyes are overexposed to light [148]. This can be resolved by limiting exposure to light, such as performing experiments in a darkroom and only applying excitation light during signal acquisition. With illumination levels less than 100 mW/cm^2, optical signals have been measured for hours with essentially no deterioration [149]. On the other hand, Windisch et al [147], using an argon ion laser with an output of 90-200 mW/cm^2 for excitation, noted that over a period of 45 minutes the magnitude of the fluorescence change decreased by approximately 20 – 25%. They were able to relate this to the duration of perfusion, and not light exposure, therefore dye washout was the reason for signal decay.

1.5.2.3 Upstroke Velocity in Optical Action Potentials

Numerous experiments using optical and microelectrode techniques simultaneously have demonstrated that optical recording gives reliable and accurate action potential signals which, in some instances, may be superior to intracellular action potentials [150]. The optical signal represents a change in flourescence relative to background flourescence (i.e. $\Delta F/F$) over the time course of the action potential. The first derivative of this signal can be used to estimate maximum rise rate from $d(\Delta F/F)/dt_{max}$. It has been shown by several investigators that the maximum rise time of the fluorescence signal is directly proportional to, and occurs at the same time as, the maximum upstroke velocity of action potentials recorded conventionally (i.e. $d(\Delta F/F)/dt_{max} \propto dV/dt_{max}$) [151-153]. In fact, the upstroke phase of optical action potentials possess the added advantage of not being contaminated by the local electrogram.

1.5.2.4 Action Potential Duration of Optical Signals

A major limitation with optical measurement techniques in cardiac tissue is the presence of motion artifacts that arise due to the mechanical effects of contraction. While the action potential upstroke is not affected by motion (it precedes mechanical systole by approximately 100 msec) the plateau and final repolarisation phase of the action potential are often obscured. Attempts to minimise contraction using low-Ca solutions [154], Ca-channel blockers, or drugs such as 2,3-butanedione monoxime [155] were partially successful. However, the signals thus obtained could not be assumed to be a direct reflection of physiologically normal processes. However, it was found that stabilising the heart against a plexiglass viewing window using a plexiglass support attached to a moveable piston essentially eliminated motion artifacts without compromising the integrity of the signal or normal cellular function [153].

Action potential duration (APD) is measured in units of time beginning from the start of the maximum upstroke phase of depolarisation to a certain level of repolarisation (i.e. 25%, 50%, 90%) based on distance from the plateau to baseline [156]. In optical signals the start time is the maximum point of the first derivitive, $d(\Delta F/F)/dt_{max}$. In guinea pig ventricular action potentials, the second derivative of the repolarisation phase may be used to estimate repolarisation time [151], which is generally considered to be equivalent to 90% repolarisation (APD_{90}) as measured conventionally [150]. However, in atrial or in rat ventricular action potentials there is no plateau from which to determine percent repolarisation, nor is it possible to determine the second derivative accurately due to the rapid exponential nature of decay. In these signals, therefore, the amplitude is used as the maximum from which % repolarisation is calculated, for both conventional microelectrode and optical recordings.

1.5.3 Correlation Analysis

Correlation analysis was used in this thesis to explore relationships between indices of antiarrhythmic protection and electrophysiologic effects in normal and ischaemic tissue. The specifics and limitations of correlation analysis will be therefore be discussed in this section.

While regression analysis considers the relationship between a dependent variable and an independent variable, correlation analysis looks for relationships between variables without making assumptions about whether they are functionally dependent on one another. Correlation analysis involves sampling two distinct variables from a bivariate normal distribution [129]. The correlation coefficient, r, describes the nature of the relationship and its significance. Thus r may be positive or negative, and values range between zero (no correlation) and one (or negative one). The correlation coefficient has no units, because it does not express the measure of change of one variable with respect to another, but rather measures the magnitude of the association. The value, r, is actually an estimate of the correlation coefficient in the bivariate population that was sampled, defined as ρ. Thus, correlation analysis tests the null hypothesis that $\rho = 0$. Results of correlation analysis, specifically the correlation coefficient r, can be tested for significance by comparing it to a critical value, $r_{\alpha(2),v}$.

Calculation of the correlation coefficient assumes that both variables are normally distributed [129] but the coefficient is fairly robust for departures from normality. The non-parametric correlation test uses the Spearman rank correlation coefficient, r_S. The absolute minimum number of data required to perform correlation analysis is $n = 3$, however this is associated with very high r values for significance and it is not recommended that correlations be attempted when $n < 4$ [129]. It should be noted that a positive correlation does not prove a causative relationship between two variable, thus examination of the data is necessary before drawing conclusions about the nature of the association.

CHAPTER 2: METHODS

All protocols performed in this thesis adhere to the standards of, and were approved by, the Animal Care Committee of the University of British Columbia.

2.1 The Test Compounds

The six drugs chosen for this thesis are from a series of chemically related arylbenzacetamides. They are: (±)-1,2-*trans*-[1-(acetoxy)-2-(4-morpholino)-2-(cyclohexane) monohydrochloride (**RSD1031**), (±)-*trans*-[2-(4-morpholinyl)-cyclohexyl]-2-(1-naphthyl) propionate hydrochloride (**RSD1030**), (±)-1,2-*trans*-1-(4-bromophenylacetoxy)-2-(4-morpholino) cyclohexane monohydrochloride (**RSD1020**), (±)-*trans*-*N*-methyl-*N*-[2-(1-pyrrolidinyl)-cyclohexyl]-3,4-dichlorocinnamamide hydrochloride (**RSD995**), (±)-*trans*-*N*-[2-(1-pyrrolidinyl)]-cyclohexyl] (3,4-dichlorophenoxy) acetamide hydrochloride (**RSD988**), and (+) - *trans* - *N*-methyl – *N* - [2-(1-pyrrolidinyl)-cyclohexyl] - benzo[b] – thiophene – 3 - acetamide hydrochloride (**RSD944**). The structures are shown in Appendix D.

2.2 Coronary Occlusion in Rats

2.2.1 Protocol

Male Sprague-Dawley rats (from UBC Animal Care), ranging in weight from 200-300 g, were randomly assigned to control or treatment groups. Rats were anaesthetised with 65 mg/kg pentobarbitone (i.p.). The trachea, carotid artery, and jugular vein were cannulated for artificial ventilation, monitoring blood pressure, and administration of drug or vehicle, respectively. The chest was opened at the fifth intercostal space, and the heart exposed for implantation of a cardiac occluder around the descending branch of the left coronary artery. Occluders were made of PE-10 tubing threaded with 5-0 surgical suture. The chest was closed using 3-0 surgical

sutures and aspirated using a 5 cc syringe to remove excess air. Body temperature was monitored using a rectal thermometer (Becton-Dickenson, Canada) and maintained between 33 and 36°C by means of a heating lamp. Animals were ventilated with humidified oxygen (stroke volume approx. 10 mL/kg/min) from the time the chest was opened until the end of the experiment.

Three pin-style ECG electrodes were implanted subcutaneously: one in the right neck region, one on the chest near the ventricles, and one in the right femoral region. Blood pressure and ECG were recorded using a Grass polygraph (Grass Instruments, USA). Recording began upon completing surgery. Blood pressure was allowed to stabilize before continuing with the experiment. Any animals exhibiting serious arrhythmias (i.e. VT or VF) during this time were excluded from the study. A sample of arterial blood was taken via the arterial cannula and potassium ion concentration was measured using an Accumet model 25 pH/ion meter (Fisher Scientific, USA). Animals were excluded from the study if the potassium concentration fell outside the range of 2.5 - 4.0 mM since variations in serum potassium can affect the incidence and severity of arrhythmias due to occlusion [157;158].

Drug or vehicle was infused for 5 minutes before (and for 15 minutes after) the occluder was pulled and sealed tight by melting the end of the tubing with a disposable lighter. Data was recorded for 15 minutes or until death or non-reversible ventricular fibrillation (> 3 min.) occurred. A sample of arterial blood was collected after the experiment (if possible) and blood potassium measured again. Arrhythmias were counted and a score assigned according to the occurrence, severity, and duration of arrhythmic events.

The arrhythmia score (AS) used in this experiment was based on that described by Johnston et al [159] and is shown in Table 2. This is a normally-distributed variable that allows for parametric statistical testing and is therefore likely to be a more sensitive method of quantifying arrhythmias than non-parametric analysis of binomially distributed raw data such as the incidence of ventricular fibrillation. Curtis and Walker [160] showed that arrhythmia scores

have higher precision and accuracy compared to raw arrhythmia data, and greater power allowing for significant results with smaller group sizes.

After the experiment the heart was removed and perfused by the Langendorff technique with saline followed by cardiogreen dye (dissolved in saline). With the occluder still attached, the dye stained only perfused tissue whereas the occluded zone was not stained. The atria were removed and the ventricular tissue was cut along the boundary of the stain and weighed; occluded zone size was expressed as a percentage of total ventricular mass. Animals were excluded from the study if this value was below 25% or above 50% as this will affect the incidence and severity of arrhythmias following occlusion [159]. For each drug 7 doses were tested with n=5 rats per dose. There were also 23 control rats.

Table 2. Arrhythmia scoring system used for coronary occlusion experiments.

Arrhythmia Score	Arrhythmic Events
0	0 – 49 PVCs
1	50-499 PVCs
2	>499 PVCs and/or 1 episode of spontaneously reverting VT or VF
3	>1 episode of VT or VF or both (<60 sec total combined duration)
4	VT or VF or both (>60 sec total combined duration)
5	Fatal VF starting at between 4 min and 14 min, 59 sec after occlusion
6	Fatal VF starting at between 1 min and 3 min, 59 sec after occlusion
7	Fatal VF starting > 1 min after occlusion

Modified arrhythmia scoring system based on that of Johnston et al [159]. Scores were assigned based on arrhythmic events following occlusion of the left coronary artery in anaesthetised rats, up to 15 minutes following occlusion. Modifications were made to satisfy requirements of regression analysis. PVC = premature ventricular contraction VT = ventricular tachycardia VF = ventricular fibrillation

2.2.2 Statistical Analysis

2.2.2.1 Analysis of Variance (ANOVA)

Before analysing dose-response curves for AS it was established that the different sets represented different populations; in other words, that each set of data for a particular dose represented a sample taken from that dose-population. ANOVA was used to test the null hypothesis that all sample means were equal (i.e. all samples were taken from the same population). If the variance within each group was greater than the variance between each group then comparing groups, or examining relationships among these groups, was determined to be invalid. ANOVA was performed using Microsoft Excel version 7.0 and significance ($p<0.05$) was determined using an F-test. No further analysis was performed on data which failed ANOVA.

2.2.2.2 Non-linear Regression

Dose-response data that passed the above ANOVA test were analysed using the Math function of SlideWrite Plus (v. 4.0) for non-linear regression analysis with replication. Arrhythmia score data was plotted on a log-scale graph whereby x = log(dose). Log-transformation of the independent variable, x, does not affect distribution of the dependent variable, y, and is therefore permissible. This yielded estimates of potency, slope, and goodness of fit (the coefficient of determination, r^2). The logistic equation used to fit the data was based on equation 2 above:

$$y = x^{a0}/(x^{a0} + a1^{a0})$$

{Equation 3}

where a0 = slope coefficient (equal to the Hill coefficient for dose-response studies) and a1 = ED_{50}. This equation assumes a maximum effect of 1.0 and a minimum effect of 0 (effect = y).

Thus, in order to use this equation for curve-fitting to arrhythmia scores, data was transformed to express "fractional change from control" using the equation:

$$y' = (y - \text{control mean}) / (\text{maximum possible score} - \text{control mean})$$

{Equation 4}

where "*y* prime" is the fractional response (between zero and one), *y* is the arrhythmia score, "control mean" is the mean arrhythmia score for all control animals and "maximum possible score" is 0 (the highest score of protection on the arrhythmia scale; see Table 2 above).

The program used estimates of equation variables before beginning the iterative process. These estimates were based on visual examination of the data. The analysis method is fairly robust to errors in estimation due to the high number of iterations performed (up to 100). Our program used the method of least squares, selecting parameter values that result in the lowest possible value for:

$$\sum_{i=1}^{k} \sum_{j=1}^{n_i} (Y_{ij} - \hat{Y}_i)^2$$

{Equation 5}

where k=7 doses, n=5 rats per dose, and $\hat{Y}$ = the value of y which falls on the regression line. The computer ceased further iterations when the difference in the sum of squares value produced became less than 1×10^{-6} (defined as the "tolerance factor" which was set by the operator). The analysis yielded estimates of slope, potency (ED_{50}), and the coefficient of determination, r^2.

2.2.2.3 Statistical Assumptions

When performing statistical tests the assumptions inherent in the method must be considered. These were addressed as below:

First, the population of *y*-values (arrhythmia score) must be normally distributed. This was confirmed by Curtis and Walker [160].

Second, the variance of the y-values sampled from each x-value population must be equal (i.e. the populations are homoscedastic). With arrhythmia score, y-value samples tend towards heteroscedasticity because as x increases, the dose of antiarrhythmic drug increases and therefore the less likely the chances of scoring low on the scale. Data could have been log-transformed to correct this error, but analysis of the residuals showed that such a transformation was not necessary (data not shown). In other words, although a plot of the residuals indicated possible heteroscedasticity, regression analysis of the plot failed to show a significant difference from a plot of residuals exhibiting homoscedasticity (i.e. where slope = 0). Therefore log-transformation of y was not warranted.

The third assumption was that the errors in y were additive. For this reason a modified arrhythmia score system was used, as shown in Table 2. According to the method upon which it was based [159], a score of 6 results from arrhythmic death after 15 minutes post-occlusion. Since, in this experiment, data was not collected past 15 minutes post-occlusion, a score of 6 was impossible. This meant the distance between a 5 and a 7 represented one "step" in the scoring system, although mathematically it represented two. Therefore the definition of a score of 6 was eliminated, and 7 and 8 changed to 6 and 7 respectively, so that data was linearly additive.

The fourth assumption was that values of y were independent; in other words, the value of one score did not influence another. This was the case in the present experiments because each rat was scored only once.

The final assumption was that values of x were obtained without error (since x is the independent variable). This was, of course, not possible but it was assumed that errors in the x data (which would be errors involved in measuring and preparing the doses) were negligible compared to measurement errors in the dependent variable y.

2.3 Electrically Induced Arrhythmias in Rats

2.3.1 Protocol

Male Sprague-Dawley rats (source: UBC Animal Care) ranging in weight from 250-350g were assigned consecutive numbers in order of their use and randomised to treatment or control. Rats were anaesthetized with 65 mg/kg pentobarbitone (i.p.). The trachea, carotid artery, and jugular vein were cannulated for artificial ventilation, monitoring blood pressure, and administration of drug or vehicle, respectively. Teflon-coated Ag/AgCl electrodes with naked tips (0.0055" diameter; A-M Systems Inc., Carlsborg, WA, USA) were implanted approximately 3 mm apart in the apical region of the ventricle via transthoracic impalement (see Figure 1). Body temperature was monitored using a rectal thermometer (Becton-Dickenson, Canada) and maintained between 34 and 36°C (± 0.5 °C) by means of a heating lamp. Animals were ventilated (small animal ventilator, Harvard Apparatus, U.K.) with room air (stroke volume approx. 10 mL/kg/min) from the time the surgical preparation was complete until the end of the experiment. Pin-style ECG electrodes were implanted subcutaneously in the right neck region, over the ventricle, and the right femoral region in an approximate lead V3 configuration.

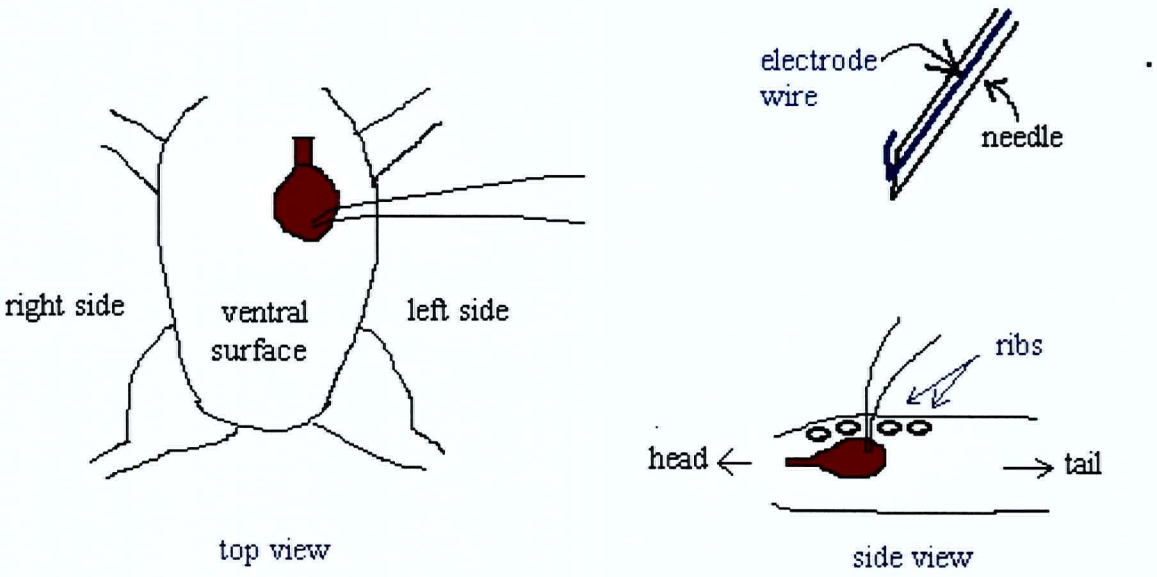
The ECG and blood pressure were monitored while the rat was allowed to recover from preparatory surgery (approximately 15 minutes). Animals were excluded from the study if blood pressure was less than 50 mmHg and/or if any severe arrhythmias (i.e. ventricular tachycardia or ventricular fibrillation) were observed during the equilibration period.

Drug solutions were made by dissolving pre-weighed samples of drug in saline according to formulae described in Appendix A. Experiments were performed according to a random and blind design with pre-weighed blinded samples of drug for five rats (per drug treatment group) and five control rats (receiving saline) randomized in a 5 x 7 table.

After the equilibration period the automated acquisition program was begun using the LabView software described below in section 3.3. The first 5-minute acquisition served as a

"pre-drug" or control period. Data were recorded for 5 minutes; during the last two minutes of the sampling period the stimulation protocol was carried out. At the start of the sampling period, following the control period, a solution of drug was infused via the jugular vein cannula (Model 22 Infusion Pump, Harvard Apparatus, USA) at 0.5 mL/hr. For each subsequent sampling period the infusion rate was increased by a factor of two. Stimulation protocols were always performed during the last two minutes of each sampling period. Seven doses and one pre-drug stage were performed for each rat (i.e. 8 sampling periods total per experiment).

Figure 1. Schematic representation of transthoracic electrode placement in electrical stimulation experiments. Electrical stimulation of the left ventricle was performed in anaesthetised rats. Stimulating electrodes were threaded through a 27½ gauge needle (upper right) which was then inserted through the fifth intercostal space (bottom right) into the base of the left ventricle (left and bottom right). Two electrodes were used for stimulation, placed approximately 2 mm apart.



2.3.2 Electrical Stimulation Protocol

Electrodes (implanted through the chest into the ventricles, as described above) were connected to a stimulator which was computer-controlled. The stimulator circuit was provided by Stopper Autolabs (Vancouver, BC, Canada) and was controlled from a Toshiba laptop computer (Satellite Pro 420CDS) via a serial port interface. The software (S.T.I.N.G., Stopper Autolabs, Vancouver, BC, Canada) controlled the pacing frequency and pulse width and allowed for the programming of various pacing protocols where one could specify frequency, pulse width, number of pulses, and premature stimulus interval. Current was adjusted manually using a suitably calibrated multi-turn linear potentiometer with maximum current output of 2 mA.

The stimulation protocol used was as follows: the threshold current for capture (iT) was determined using a 1000-pulse pacing train with 133 msec (7.5 Hz) cycle length, 0.5 msec pulse width, and variable current. Acceptable baseline iT values were between 400 – 900 μAmps. If iT fell outside this range further electrodes were implanted, and if this did not result in values within the range the animal was excluded from the study.

The effective refractory period (ERP) was determined at 0.5 x iT using the following pacing protocol: a train of 5 pulses (133 msec cycle length, 0.5 msec duration) was followed by a single extrastimulus with an interval of 30 msec (pulse width also 0.5 msec). This was followed by trains of 3 pulses each followed by an extrastimulus at increasing intervals. The premature stimulus interval was increased by 2 msec in each subsequent pacing train and was programmed to continue up to a maximum interval of 130 msec. The pacing protocol was terminated upon observation (on the ECG) that the extra pulse had generated an extrasystole.

The maximum following frequency (MFF) was determined at 0.5 x iT and 0.5 msec duration by increasing the pacing frequency at a constant rate of 1 Hz per second until capture was lost.

The final variable measured was VFt, as determined by measurement of the current required to induce ventricular fibrillo-flutter (see section 4.3). Pacing was performed at a cycle length of 20 msec (50 Hz) and a pulse width of 0.5 msec. Current was increased at a constant rate until ventricular fibrillo-flutter was observed on the ECG.

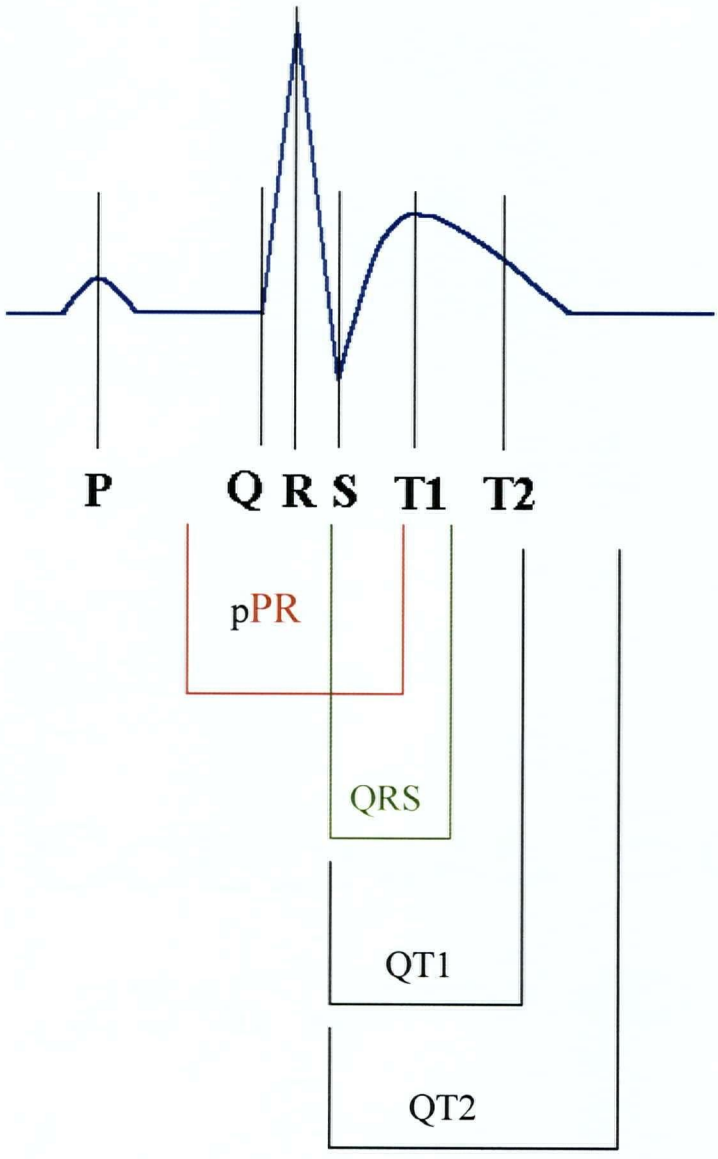
2.3.3 Data Acquisition

Blood pressure and ECG signals were amplified (ETH-260 Bridge/Bio Amplifier; CB Sciences; Harvard Apparatus, Quebec, Canada) and converted to digital signal using a National Instruments A/D board (PCI-MI0-16E-4, model TBX68, National Instruments, USA). Data was acquired continuously throughout the experiment using LabView version 4.0.1 for Windows 95 at a sampling rate of 1 kHz, on a 200 MHz Pentium computer. ECG and blood pressure signals were displayed in electronic chart form in real time. Files were divided into 5-minute consecutive sampling periods.

2.3.4 Data Analysis

For each five-minute sampling period the ECG was analysed at the three-minute mark (i.e. immediately prior to performing the stimulation protocol). Five consecutive complexes were selected between t = 177 to 180 sec. LabView determined the location of the points P, Q, R, S, T1, and T2 for the complexes selected. T1 was defined as the peak of the T-wave, and T2 as the halfway point of T-wave repolarisation from peak to baseline (see Figure 2). The program allowed for user correction of the detected points before data were recorded. The data for time of occurrence of these points (in seconds) and their magnitude (for P, R, and T1 and T2; in mV) were calculated by LabView and saved in a tab-delimited text file. This process was repeated for every sampling period for each experiment (i.e. total of 8 sampling periods per experiment).

Figure 2. ECG parameters measured in electrical stimulation experiments. During electrical stimulation experiments, the electrocardiogram was recorded three minutes into each five-minute aquisition stage. ECG parameters were measured at the points shown to yield PR Interval (red), QRS Duration (green), QT1 and QT2 Intervals (black). These points were determined using an automated analysis program and could be corrected by the user to ensure accuracy.



A section of the blood pressure recording, corresponding to the same time frame during which the analysed ECG complexes occurred, was computer analysed. The computer determined maximum systolic pressure, minimum diastolic pressure, and mean arterial pressure (MAP) within the selected time frame. MAP was graphed and fit to a dose-response curve as described above. Each dose response curve represents one rat; each point on the graph is one measurement for that dose.

Data for iT, MFF, and VFt were recorded during the experiment. Endpoints for ERP were located manually by scrolling through the ECG chart and measuring the interval between S1 and S2 for the pulse train that caused an extrasystole. Data was grouped by treatment and fit to dose-response curves as described above. Each curve represents one rat; each data point is one measurement of that parameter for each dose.

Data for iT, VFt, ERP, MFF, QRS Duration, PR Interval, QT1 Interval, QT2 Interval, and MAP were transformed according to the following equation:

$$y' = \frac{y - C}{C} \times 100$$

{Equation 6}

where y' = transformed data and C = pre-drug (control) value. Thus data was expressed as percent increase from pre-drug and plotted on a graph with dose on a log scale.

Before fitting data to dose-response curve equations, an exclusion protocol was performed as follows: data for each animal were submitted to a linear regression ($y = mx + b$) to determine if any sets of data represented a null response. In other words, if no significant change from control was observed at any dose then the data represented a straight line with slope of zero. Such data cannot be fit to a sigmoidal equation. This would be the expected result for animals in the control group. If three or more animals in a drug treated group, out of a set of five, were excluded by this method the drug was considered to have no effect on the variable

measured. If less than three animals showed zero-response, they were excluded from that group for the purposes of curve-fitting.

2.3.5 Non-linear Regression Analysis

Non-linear regression was used to fit data that passed the above exclusion test using the following equation, based on Equation 2 (see Introduction):

$$y = \max \Big/ \left[1 + \left(\frac{x}{a1}\right)^{-a2}\right]$$

{Equation 7}

Where "max" = y-max, $a1$ = ED_{50}, and $-a2$ = slope. This equation is equivalent to Equation 2 except that instead of a maximum of 1 this formula allows the user to input the maximum y-value. The minimum value is zero. The curve-fitting program (maximum 100 iterations, sum of squares tolerance $1x10^{-6}$), estimated slope and ED_{50}. Y-max was set at 100%, even though there was no evidence to suggest that 100% represented the maximum possible drug effect for most of the variables measured. However, pilot experiments showed that a change in y-max of a magnitude of 100 did not significantly alter the results of data extrapolation from the estimated curve, so long as extrapolations were performed within the range of the curve corresponding to the doses tested. Therefore, 100% was chosen for all variables. For mean arterial pressure, which represented a decreasing effect, 100% was the true maximum.

2.3.6 Potency Indices (D25%)

D25% was defined as the dose (in μmoles/kg/min) which produced a 25% change from pre-drug (i.e. the x-value for y = 25%) and was determined by extrapolation from the dose-response curve for each animal in a treatment group. These values were then averaged using a

geometric method for determination of the mean, which was applicable to this data due to the fact that a percent change from control was being averaged [161]. This method uses a logarithmic equation to determine mean and standard error, which can than be translated back into common units by taking the antilog. The result is a mean D25% for each treatment group, with a range of standard error that is not symmetrical about the mean.

Since the maximum response for most variables was not known, standard potency measures such as ED_{50} could not be used. D25% was chosen as a potency indicator since it allowed extrapolations to be contained within the data sets. The reliability of the estimate decreases as one extrapolates further beyond the data sets used to fit the curve. For many parameters drug responses did not reach the D50% level, whereas the D25% was reached for all drugs.

2.4. Isolated Heart Experiments

2.4.1 Experimental Method

2.4.1.1 Preparation

Male rats weighing between 250-350 g were randomly assigned to one of 14 treatment groups. There were two control groups, occluded and sham-occluded, and twelve drug-treated groups: low concentration and high concentration for each of the six test compounds investigated in this thesis. Each treatment group contained 5 animals.

Rats were anaesthetised with 65 mg/kg pentobarbitone (i.p.) and hearts were rapidly excised and mounted on a Langendorff perfusion apparatus and attached to an aortic cannula with surgical thread. Retrograde aortic perfusion with Krebs buffer (NaCl 118 mM, KCl 4.7 mM, glucose 10 mM, $NaHCO_3$ 25 mM, KH_2PO_4 1 mM, $MgSO_4 \cdot 7H_2O$ 1.2 mM, $CaCl_2 \cdot 2H_20$ 2.5 mM, buffered to pH 7.4 with $95\%O_2/5\%CO_2$ gas, 37°C) was applied at a constant pressure of 100 mmHg.

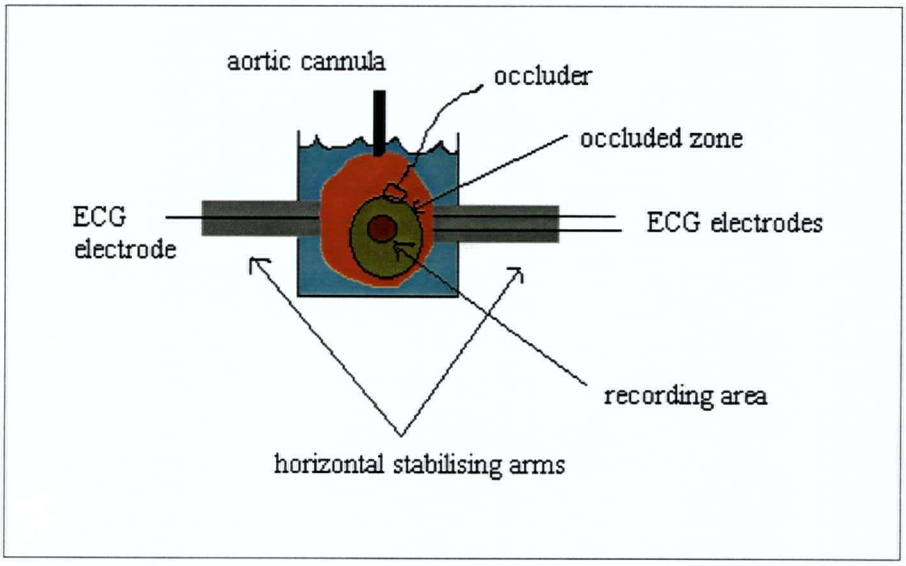
An occluder identical to that used in the whole animal occlusion experiments was placed around the left coronary artery and left loose. Two stimulating electrodes identical to those used in the electrical stimulation experiments were implanted into the apical region of the ventricle on the posterior surface of the heart approximately 1-2 mm apart.

The heart was enclosed in a plexiglass chamber that consisted of two thin plexiglass viewing surfaces on opposite faces (see Figure 3). The heart was therefore immersed in a bath of effluent that exited the chamber via an outflow tube. With proper positioning there is a microthin layer of buffer between the surfaces of the heart that are pressed up against the viewing window and the plexiglass, but there is not expected to be any appreciable exchange of this fluid with the rest of the perfusate in the chamber. This lack of fluid exchange at the viewing sites is insignificant relative to the nutrient supply provided by retrograde aortic perfusion, and would not be expected to render the tissue against the glass ischaemic nor to provide for significant oxygen exchange and nutrient washout during regional ischaemia.

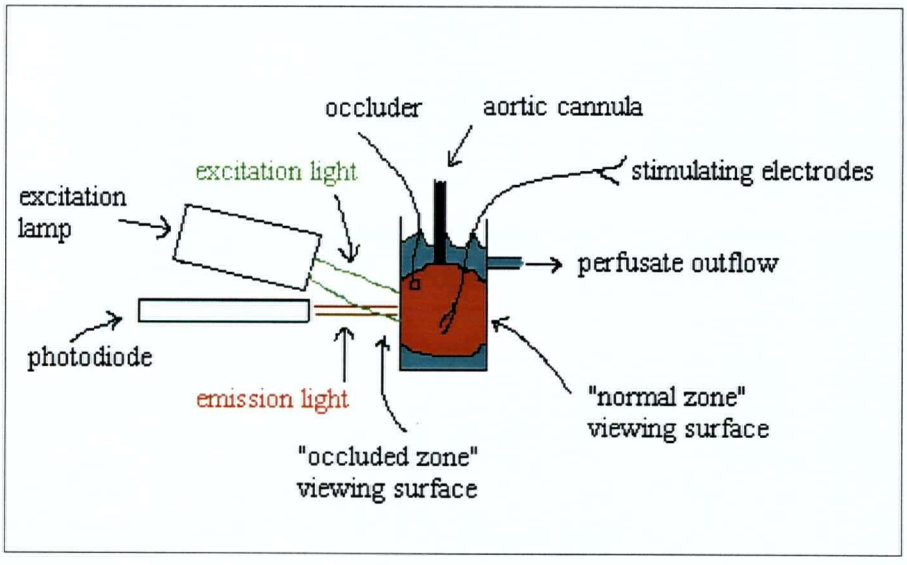
The heart was positioned such that the region which would be rendered ischaemic following tightening of the occluder was centred on one viewing surface (posterior side of heart), and the opposite side (anterior side of heart, non-occluded region) was centred on the other viewing surface. The heart was supported on the sides by means of Teflon supports into which were implanted recording electrodes. The exterior ends of these electrodes were connected to ECG leads and fed to an oscilloscope (Gould Advance model OS250B, Gould Instruments, Essex, UK) and then to the data acquisition system. The stimulating electrodes implanted in the ventricle exited the chamber through a port on the top and were connected to a Grass stimulator (Grass SD9, Grass Instruments). The end of the occluder also exited the chamber via a port so that it could be tightened during the experiment.

Figure 3: Viewing chamber for isolated heart experiments. The side view (A) shows positioning of lamp and photodiode on the ischaemic zone side of the preparation. Another lamp and photodiode positioned on the opposite face (normal zone) are not shown. The frontal view (B) shows the horizontal stabilising arms and ECG electrodes, as well as a schematic representation of the recording area within the ischaemic zone.

A



B



Two buffer reservoirs were used which could be switched during the course of an experiment. One reservoir contained plain Krebs buffer, and the other contained a solution of drug dissolved in Krebs buffer (or plain buffer in control experiments).

2.4.1.2 Optical System

Excitation light was provided by two tungsten lamps in specialised metal housings, each with its own DC power supply (BK Precision model 1686, set to 9V, 7.5 amps). One lamp was focussed on each viewing surface and an aperture on each lamp was set to allow for an illumination area of approximately 3 mm diameter. The illuminated area was focussed in the center of each viewing window. Optical filters were inserted at the illuminating end of the lamp housings to select for excitation light at 540 nm (narrow band-width filter, +/- 5 nm, Omega Optical, Vermont, USA).

Fluorescence (emission) light was detected by placing a photodiode assembly in the reflected light path of each illuminated area. Photodiode assemblies were contained in specialised electrically-shielded cylinders that were grounded. Each assembly contained one photodiode cell (silicon blue-enhanced photovoltaic photodiode, type PDB-V113, active area 7.75 mm^2, Photonic Detectors Inc., California, USA), a filter (high pass, 610 nm; Omega Optical, Vermont, USA), and a pre-amplifier. The photodiode cell current output was converted to voltage, zeroed to the relative DC offset, and sent to a second amplifier (purchased from University of Pittsburgh, Department of Cell Biology and Physiology, Pennsylvania, USA). The amplifier allowed for setting the gain, autozeroing baseline, and anti-alias filtering. Signals from the amplifier were fed to the data acquisition system.

2.4.1.3 Data Acquisition

Data was acquired using a Pentium 266 MHz computer. Channels for each of two optical signals and perfusion pressure were fed to an A/D board (DAQ card PCI-MIO-16E-4, model TBX68, National Instruments, USA) and then to the computer. LabView version 4.0.1. for Windows (National Instruments) was used to acquire and display signals in real time. Data was sampled at 3 kHz and stored on the hard drive for subsequent analysis. The ECG was monitored on an oscilloscope during the experiments.

2.4.1.4 Experimental Protocol

Hearts were paced at 5 Hz, pulse width 1.0 msec, and 1.5x threshold current for capture throughout the experiment and for 15 minutes prior to beginning the experiment (equilibration period). Dye was infused by administering a slow bolus injection of 20 μL of a 2 mM solution of di-4-anepps (Molecular Probes Inc., USA) into the perfusate. Dye was allowed to equilibrate for 10 minutes prior to beginning the experiment. During this time, fine focussing was achieved by adjusting the focal distance of the lamps and the position of the diodes while monitoring the optical action potential signals. If motion artifacts were present, the chamber was adjusted to minimise motion of the heart against the viewing windows.

Once dye was infused, all further procedures were performed in the dark to minimise optical signal contamination with ambient light. The computer monitor was in an adjoining room with a door that could be shut during signal acquisition. A sample of action potentials was recorded by turning on the tungsten lamps and recording with LabView for a period of 3 to 5 seconds. Samples were acquired at the start of the experiment. Immediately following this baseline recording, drug infusion was begun by switching to the drug/buffer reservoir. Two concentrations of drug were used, one per experiment. Concentrations were selected based on ischaemia-selectivity data provided by Nortran Pharmaceuticals and on pilot experiments. Drug

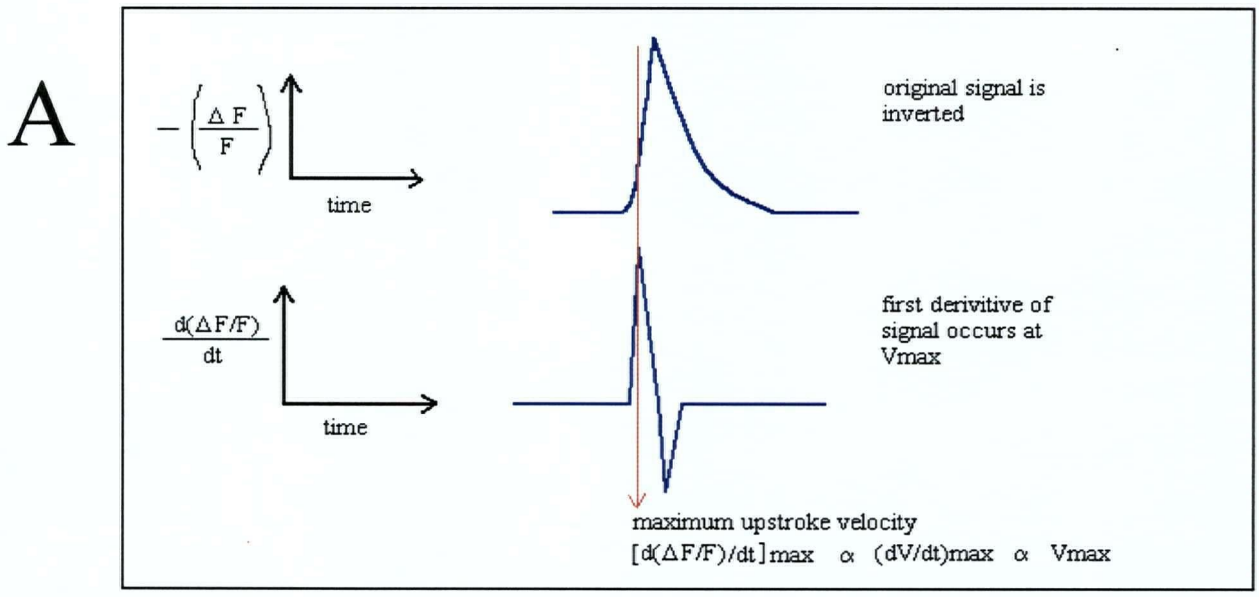
was perfused for five minutes and the occluder tightened. Immediately following occlusion, optical signals were recorded every minute for 5 minutes, or until irreversible VF occurred (> 3 min duration).

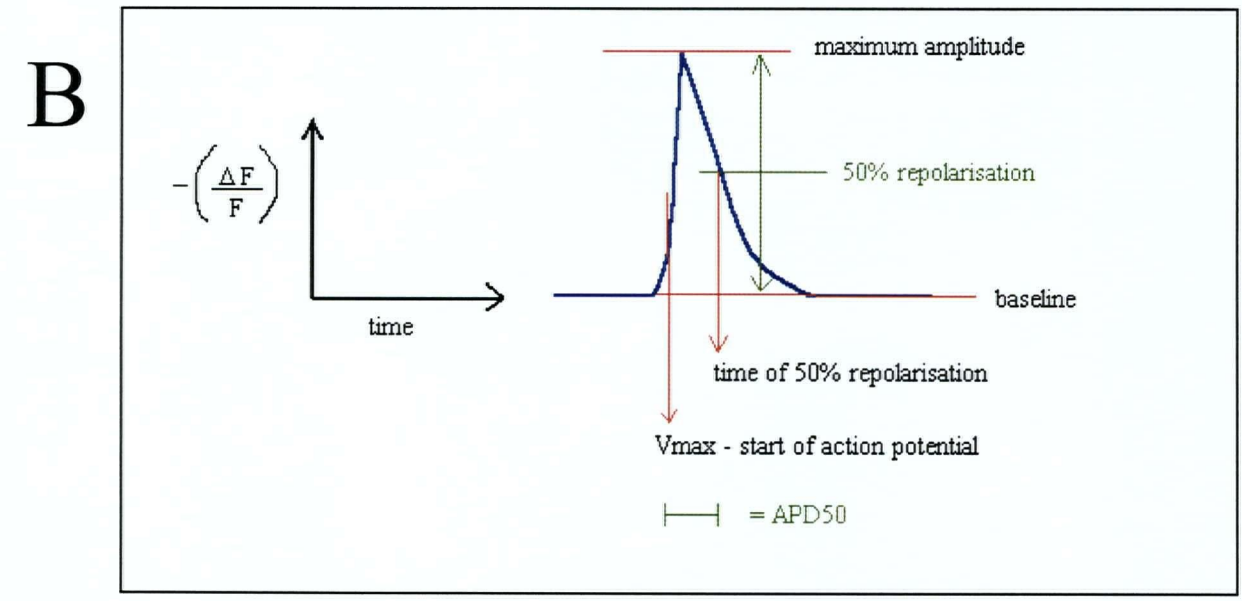
At the end of the experiment hearts were tagged with an electrode wire to identify the illumination zone (one on each side) and then stained (see procedures for Coronary Occlusion experiments above) to confirm that optical signals were recorded from occluded and perfused zones, respectively. The size of the occluded zone was measured by calculating the percent mass of occluded ventricle. If the occluded zone was less than 25%, or greater than 50%, that experiment was excluded, consistent with exclusion criteria for acute regional ischaemia in whole animals (see Coronary Occlusion experiments above).

2.4.1.5 Data Analysis

The time point during ischaemia at which action potentials were analysed for drug effects in normal and ischaemic tissue was chosen as three minutes post-occlusion. The reasons for choosing this particular time were based, in part, on the results of pilot experiments and will be discussed in the Results section. Optical signals from pre-drug and 3 minutes post-occlusion periods were analysed for each of the two sites (ischaemic and normal zones). A group of 5 action potentials were selected from each period and analysed for $d(\Delta F/F)/dt_{max}$ and APD_{25}, APD_{50}, APD_{75}, and APD_{90}. Briefly, signals were smoothed (Inverse Chebychev algorithm; 150 Hz low-pass frequency), inverted, and the first derivative was calculated for each of the five selected action potentials. The first derivative corresponded to the maximum rate of change in fluorescence (see Figure 4a). The time at which this point occurred was taken to be the start time from which action potential durations were calculated. APDs were determined based on percent repolarisation from the point of maximum upstroke (ie. amplitude) to baseline (see Figure 4b).

Figure 4. Determination of point of maximum upstroke and action potential duration in optically recorded action potentials. Signals were smoothed and inverted before analysis. A) the first derivitive was calculated and the maximum was taken as the start time of the action potential. This point, $d(\Delta F/F)/dt_{max}$, is directly proportional to V_{max} as described in the equation. B) action potential duration (msec) was calculated as percent repolarisation using the point of Vmax as start time and the point of % repolarisation as end time (shown is APD_{50} as an example). Percent repolarisation was determined using the distance between maximum amplitude and baseline.





The points of maximum upstroke velocity, $d(\Delta F/F)/dt_{max}$, and APDs for occluded and ischaemic zone action potentials were averaged for each group of five signals. Data was expressed as percent change from pre-drug using the following equation:

$$y = [(\text{post-occlusion} - \text{pre-occlusion})/\text{pre-occlusion}] \times 100$$

{Equation 8}

Because $d(\Delta F/F)/dt_{max}$ is directly proportional to dV/dt_{max}, the percent change in one is equal to that of the other. Therefore *y* was expressed as percent change in V_{max}, or "ΔV_{max}". An example of how ΔV_{max} was calculated can be found in Appendix C. APDs were expressed as "ΔAPD".

2.4.1.6 Signal Fidelity

Signal-to-noise ratios (SNRs) were determined by standard methods [153]. The mean maximum amplitude of a group of consecutive action potentials was determined and divided by the standard deviation of a group of data points recorded during diastole to yield a signal-to-noise ratio, SNR. This ratio was determined at baseline and at 1-minute intervals following occlusion, since action potential amplitude decreased significantly over time due to ischaemia.

A minimum acceptable SNR value of 40 was chosen based on the results of ANOVA testing. The ANOVA was performed to test for differences between the means of action potential amplitude, and amplitude of points sampled during diastole (i.e. baseline). This test showed that at SNRs less than 40 it was often not possible to significantly distinguish between these two populations.

2.4.2 A Quantitative Measure of Ischaemia-Selectivity

A quantitative index of selectivity of drug action for ischaemic versus normal tissue was determined for each of the two electrophysiologic properties measured: decrease in rise rate of the action potential and prolongation of repolarisation. An "ischaemia-selectivity index" (ISI) was determined for both of these variables, for each compound in this study. ISI was calculated for ΔV_{max} (ISI-V) and ΔAPD (ISI-R, for repolarisation) using three different techniques, as described below. These methods were later evaluated based on correlation analyses.

For ISI-R, APD_{50} was used in all calculation methods. APD_{90} was not used due to motion artifacts which often obscured this portion of the action potential. While motion artifacts obscured APD_{75} to a far lesser extent there were still a few experiments where this value could not be accurately determined, whereas APD_{50} could be measured accurately in every experiment. Furthermore, effects on APD_{50} were usually greater than effects on APD_{75} and APD_{90}, thus providing a more sensitive measure of ischaemia-selectivity.

2.4.2.1 Response Ratio Method

ISI was determined using the ratio of responses produced by the highest concentration of drug in ischaemic versus normal tissue. The change in ΔV_{max} and ΔAPD produced by drug was determined for normal and ischaemic tissue and, because control experiments showed a significant effect of ischaemia on these parameters, control values were subtracted from drug-treated values. The ratio of the resulting values was determined (ischaemic/normal) such that a value greater than one represented stronger effects in ischaemic tissue relative to normal tissue. A value less than one indicated effects were stronger in normal tissue than in ischaemic tissue.

Eg. ISI-V (Response Ratio Method) =

$$\frac{[\Delta V_{max} \text{ for drug (in ischaemic tissue)}] - [\Delta V_{max} \text{ for control (in ischaemic tissue)}]}{[\Delta V_{max} \text{ for drug (in normal tissue)}] - [\Delta V_{max} \text{ for control (in normal tissue)}]}$$

{Equation 9}

2.4.4.2 Parallel Shift Method

ISI was also calculated as a potency shift of the normal dose-response curve by the conditions of ischaemia using the 2 x 2 parallel shift bioassay equation. In this method slope was assumed to be equal for both dose-response curves (normal and ischaemic) and did not factor directly into the calculations. Such calculations require at least two doses of drug and assume that the responses to both doses fall on the linear portion of the dose-response curve. The formula used to determine potency shift was:

$$ISI = \{[(N_H + N_L) - (I_H + I_L)] / [(N_H + I_H) - (N_L + I_L)] \times \log (H/L)\} (-1)$$

{Equation 10}

where N_H = response in normal zone, high concentration; N_L = response in normal zone, low concentration; I_H = response in ischaemic zone, high concentration; I_L = response in ischaemic zone, low concentration; H = high concentration of drug (in μM), and L = low concentration of drug (in μM). The formula for a two-by-two assay is generally used to calculate right shifts of dose-response curves when an antagonist agent is present. Thus the above equation was multiplied by –1 to specifically calculate the leftward shift of the curve from normal to ischaemic conditions. This formula was used to calculate ISI for both conduction and repolarisation effects (ISI-V and ISI-R). A positive ISI value indicated greater potency in ischaemic tissue, whereas a negative ISI value indicated greater potency in normal tissue.

2.4.6.3 Potency Shift Method

The assumption of equal slopes in the above method was not always appropriate because responses for some drugs did not fall on the linear portion of the dose response curve. For this reason ISI was calculated using another method. From drug data in normal or ischaemic tissue that clearly showed a separation of effect between low and high concentration (i.e. both produced a significant effect relative to controls) the slope was determined and the mean slope calculated for ΔV_{max} and ΔAPD_{50}. These mean slopes were then used to determine a potency shift by first calculating "b" from the linear equation $y = mx + b$ for normal and ischaemic data using the high concentration as x, response as y, and mean slope as m. The intercept b was then substituted into the equations for normal and ischaemic data and x was determined for both at a given y. The ratio of these x values yielded a potency shift ratio for ΔV_{max} or ΔAPD_{50}. A positive value indicated that drug was more potent in ischaemic tissue whereas a negative value indicated greater potency in normal tissue.

2.5 Correlation Analysis

Correlation analysis was performed to test for significant relationships between various indices of drug action measured in this study. These indices are listed in Table 3. Since ISI-V and ISI-R were determined using three different methods, correlations were performed on values from the method that was determined to yield the most accurate estimates of ischaemia-selectivity (see Results). Correlations were analysed for significance by comparing correlation coefficients with critical values at $p < 0.05$. The degrees of freedom, ν, is equal to n minus 2 therefore the critical value was: $r = 0.811$.

Table 3. Variables used in correlation analysis

Variable	Description
ED_{50}	Potency against ischaemia-induced arrhythmias
slope	Slope of dose-response curve for protection against ischaemia-induced arrhythmias
r^2	Goodness of fit of dose-response curve for protection against ischaemia-induced arrhythmias
D25% for VFt	Potency index for protection against electrically-induced arrhythmias
D25% for MAP	Potency index for hypotensive effects
D25% for ERP	Potency index for increase in effective refractory period
D25% for PR	Potency index for prolongation of the PR Interval of the ECG
D25% for ERP	Potency index for prolongation of the effective refractory period
D25% for MFF	Potency index for decrease in maximum following frequency
D25% for QT1	Potency index for prolongation of QT1 Interval
D25% for QT2	Potency index for prolongation of QT2 Interval
ISI-V	Ischaemia-selectivity index for effects on excitability (V_{max})
ISI-R	Ischaemia-selectivity index for effects on repolarisation (APD)

CHAPTER 3: RESULTS

3.1 Coronary Occlusion Experiments

The incidence of one or more episodes of VT and VF in vehicle treated (control) animals was 23/23 and 19/23, respectively. Mean arrhythmia score and standard error of the mean for control animals was 5.2 ± 0.26 (n=23). The upper and lower 95% confidence intervals were 5.7 and 4.7, respectively. This translates to 0.1 and - 0.1 (and mean = 0) when the data is transformed using the formula described in Methods.

Table 4 shows the incidence of VT and VF for each drug treatment group. In the RSD988 group, at the highest dose of 8 μmol/kg/min, three animals died of cardiovascular collapse. Therefore, *n* for this group was 3.

Arrhythmia scores for each treated group were subjected to ANOVA testing. For every treatment group the null hypothesis was rejected ($p < 0.05$) allowing us to proceed with non-linear regression analysis (ANOVA statistics may be found in Appendix B). The results of non-linear regression analysis are shown in Figure 5 and Table 5. Parameter estimates from curve fits include: the coefficient of determination for the regression (r^2), slope of the dose response curve, and ED_{50} for protection against ischaemia-induced arrhythmias.

All drug-treated groups showed a dose-dependent reduction in arrhythmia score except RSD944. For this drug, the mean arrhythmia score was significantly greater than that of control rats for six out of seven doses tested, but there was no apparent dose-relationship. Thus the data for this group failed the non-linear regression and slope and ED_{50} could not be determined for this compound using regression analysis.

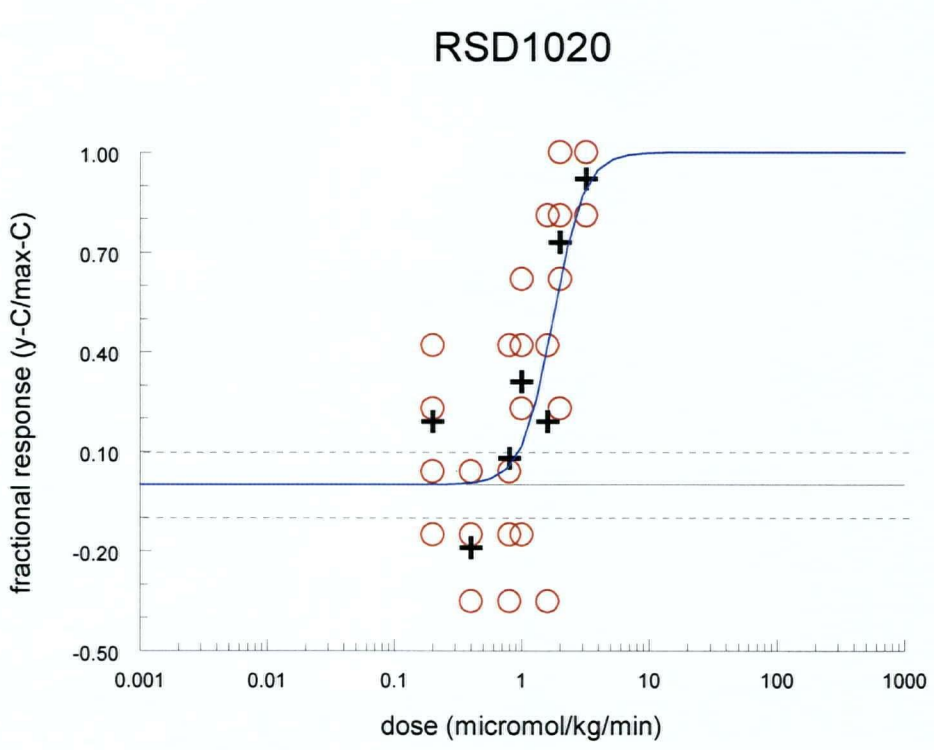
Table 4: Incidence of ventricular tachycardia and ventricular fibrillation in drug-treated groups in coronary occlusion experiments.

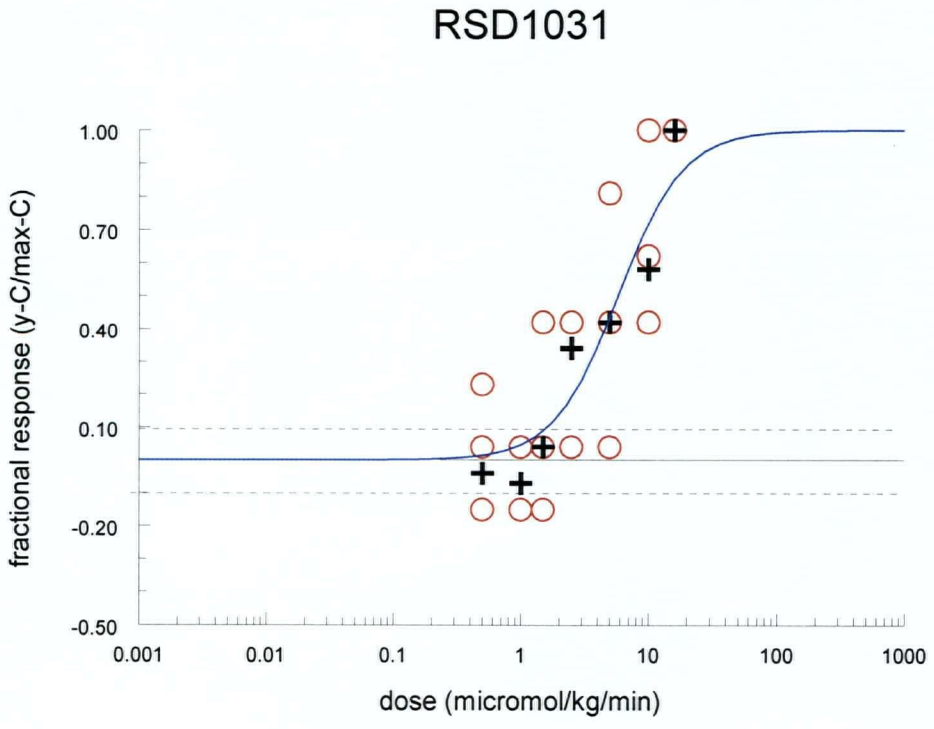
Dose	VT	VF	Dose	VT	VF	Dose	VT	VT
RSD1031			**RSD1030**			**RSD1020**		
0.5	5/5	5/5	**0.05**	5/5	5/5	**0.2**	5/5	5/5
1.0	1/5	5/5	**0.25**	3/5	2/5	**0.4**	5/5	5/5
1.5	4/5	4/5	**0.5**	4/5	5/5	**0.8**	5/5	4/5
2.5	4/5	2/5	**1.0**	5/5	3/5	**1.0**	5/5	4/5
5.0	3/5	1/5	**2.5**	5/5	1/5	**1.6**	4/5	4/5
10	0/5	0/5	**5.0**	2/5	0/5	**2.0**	2/5	2/5
16	0/5	0/5	**10**	1/5	0/5	**3.2**	0/5	0/5
RSD995			**RSD988**			**RSD944**		
0.05	5/5	3/5	**0.1**	3/5	3/5	**0.05**	4/5	2/5
0.125	5/5	4/5	**0.5**	4/5	1/5	**0.1**	4/5	3/5
1.0	2/5	1/5	**1.0**	3/5	1/5	**0.2**	5/5	2/5
1.5	1/5	0/5	**2.0**	4/5	1/5	**0.3**	4/5	0/5
2.0	4/5	0/5	**3.0**	3/5	1/5	**0.4**	4/5	5/5
3.0	1/5	0/5	**4.0**	1/5	0/5	**0.8**	4/5	2/5
4.0	1/5	0/5	**8.0**	0/2*	0/2*	**1.0**	2/5	0/5

Incidence of ventricular tachycardia (VT) and ventricular fibrillation (VF) per *n* rats in each drug treatment group. Shown are data for six test compounds, seven doses each. Doses are in μmol/kg/min. * in this group 3 rats died of cardiac output failure.

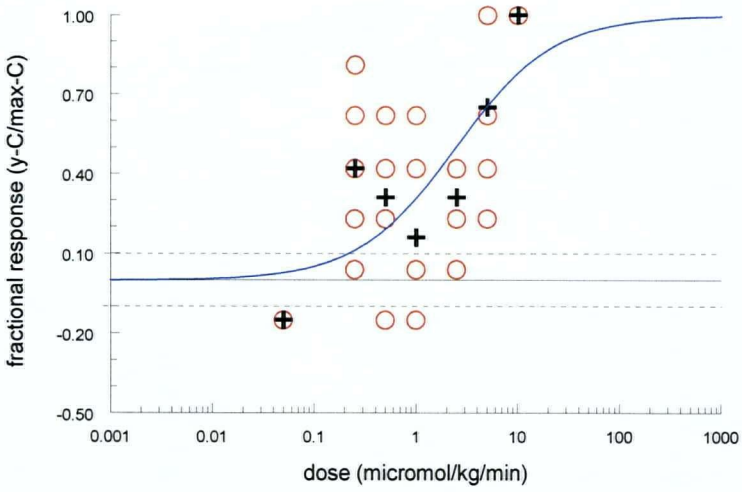
Figure 5: Arrhythmia score data and fitted curves for treatment groups in coronary occlusion experiments. Figures are grouped according to results: A) RSD1020 and RSD1031 had goodness of fit values greater than 0.5 and slopes greater than 1; B) RSD1030, RSD995, and RSD988 had slopes less than 1 and goodness of fit values less than 0.5; C) RSD9444 could not be fit to the regression equation. Arrhythmia scores were expressed as fractional response (see Equation 4) and were negative when AS in the treated group was more than C. Fractional response data for individual animals (red circles) were fit to Equation 3 using non-linear regression. Curves of best fit are shown in blue. There were five animals per dose (except for the highest dose of RSD988). Some scores occurred more than once within a group and their symbols overlap, therefore mean arrhythmia scores for each dose (black crosses) are shown for reference. Mean scores were not used for the regression. Also shown on each graph are mean arrhythmia score for vehicle-treated animals (solid black horizontal line) and 95% confidence intervals for the mean (dashed black horizontal lines).

A


RSD1020
fractional response (y-C/max-C)
1.00
0.70
0.40
0.10
-0.20
-0.50
0.001
0.01
0.1
1
10
100
1000
dose (micromol/kg/min)

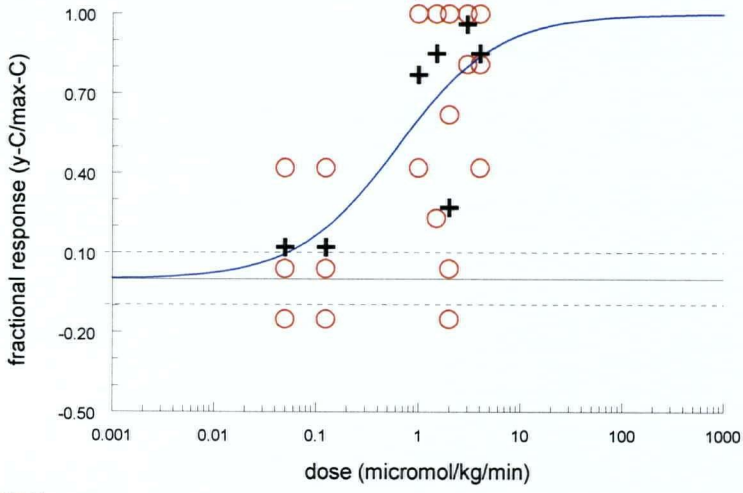

RSD1031
fractional response (y-C/max-C)
1.00
0.70
0.40
0.10
-0.20
-0.50
0.001
0.01
0.1
1
10
100
1000
dose (micromol/kg/min)

RSD1030

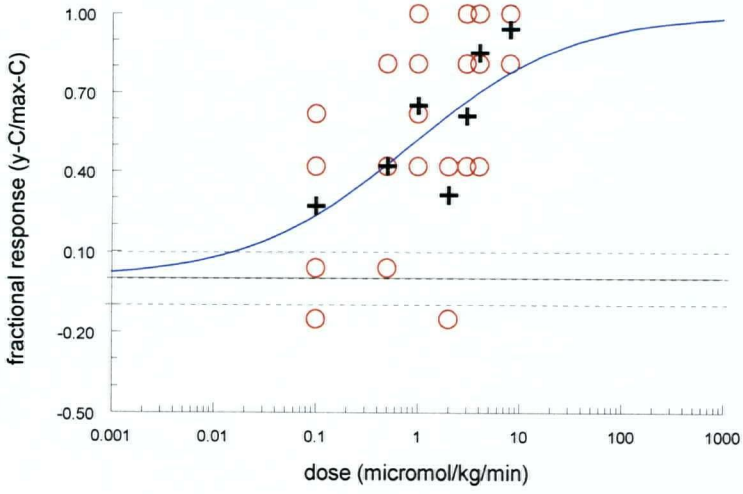


B

RSD995



RSD988



C

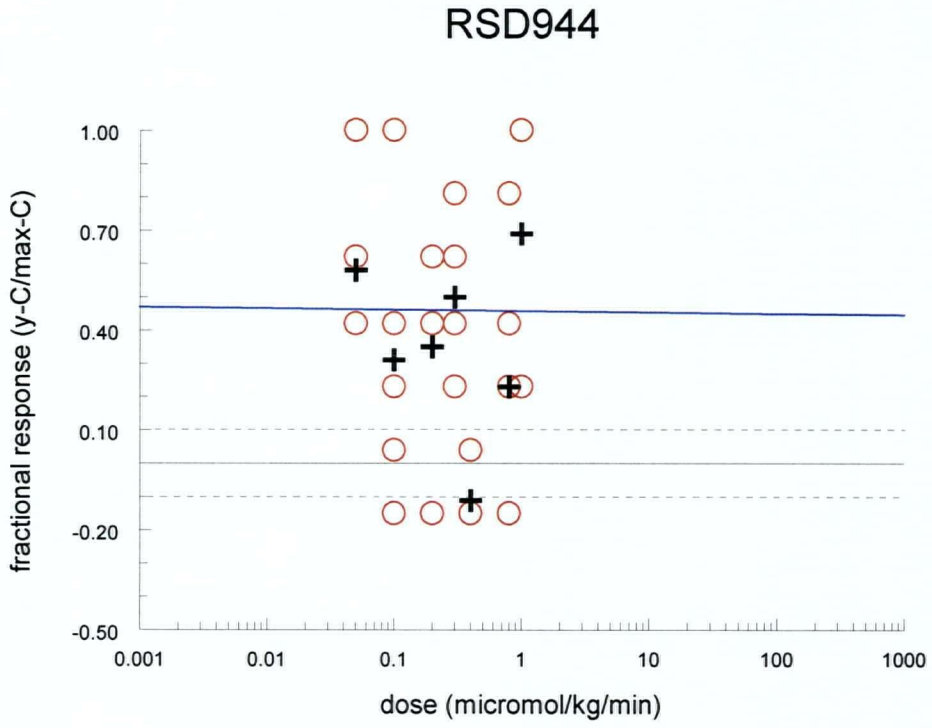

RSD944
fractional response (y-C/max-C)
1.00
0.70
0.40
0.10
-0.20
-0.50
0.001
0.01
0.1
1
10
100
1000
dose (micromol/kg/min)

Table 5: Non-linear regression statistics for coronary occlusion experiments

Compound	r^2	p	Estimate of Slope ± Std. Error	Estimate of ED_{50} (μmol/kg/min) ± Std. Error
RSD1031	0.702469	< 0.0005	1.73 ± 0.37	5.80 ± 0.82
RSD1030	0.445011	< 0.0005	0.91 ± 0.28	2.46 ± 0.79
RSD1020	0.504904	< 0.0005	3.58 ± 1.49	1.77 ± 0.20
RSD995	0.37926	< 0.0005	0.89 ± 0.32	0.62 ± 0.28
RSD988	0.267188	< 0.0025	0.55 ± 0.20	0.85 ± 0.37
RSD944	0.058199	> 0.05	NE*	NE*

Curve fit parameters and fit statistics from non-linear regression analysis of antiarrhythmic dose response curves from coronary occlusion experiments. The coefficient of determination for the regression (r^2), slope, and ED_{50} were estimated by fitting to Equation 4 (see section 2.2.2.2). Significance of the regression was determined using an F-test (ANOVA). Critical value = $F_{\alpha(1),1,n-2}$. *NE = not estimatable

RSD1031 and RSD1020 produced steep, unambiguous dose-response curves with all data points lying along the line of best fit. RSD1030, RSD995, and RSD988 showed more scatter about the fitted curves and the slopes were lower. For RSD944 there was no discernable dose-response curve, although there was a scattering of data points with some animals having no arrhythmias. These points did not appear to correlate with dose.

Correlation analysis of variables from this experiment showed a significant inverse relationship between ED_{50} and r^2 ($r = -0.863$ vs. $r_{critical} = 0.811$, $p < 0.05$). Thus those compounds with greater potency showed less goodness of fit to the regression equation, whereas goodness of fit and slope tended to increase together.

Rank order of variables:

slope	**1020 > 1031 > 1030 > 995 > 988 > 944**
goodness of fit (r^2)	**1031 > 1020 > 1030 > 995 > 988 > 944**
potency (ED_{50})	**944* > 995 > 988 > 1020 > 1030 > 1031**

*944 could not be determined directly since it did not fit the regression equation. However, estimation of ED_{50} by eye suggests that it is the most potent of all compounds studied in terms of the dose that produced a half-maximum response (see Figure 5C).

3.2 Electrical Stimulation Experiments

3.2.1 Linear Regression Analysis

Control experiments (vehicle alone) were performed to determine if there were any time-dependent effects on the variables measured. Linear regression showed that while some slopes differed significantly from zero, estimated slopes were still very close to zero, and data did not fit a dose-response equation. Furthermore, such data never constituted the majority of animals in any control group for any response. It was concluded, therefore, that there were no significant time-dependent effects for any of the variables measured. Thus, all animals served as their own controls (using values observed in pre-drug measurements).

Linear regression was used to test drugs which appeared to lack dose-related effects. RSD995 showed no effect on mean arterial pressure (MAP) since all animals failed the linear regression test. This was therefore determined to indicate a true lack of effect of RSD995 on MAP at the doses tested.

Animals showing zero drug effect for a variable were excluded from analysis for that variable. Only two animals were rejected using this technique, one in the RSD1031 group for effects on mean arterial pressure, and one from the RSD1030 group for effects on PR Interval. Visual examination of the data indicates that these could be explained by biological variability, and not an error in experimental technique. In the case of RSD1030 effects on PR Interval were very small and it was only at the highest dose that response deviated significantly enough from baseline to produce a negative result in linear regression (i.e. slope significantly different from zero). For one animal, this response fell just short of that needed to produce a significant effect above baseline (relative to variability of the data about the mean), and therefore it produced a positive result in linear regression (slope not different from zero).

The case was similar for the animal excluded from the RSD1031 group for effects on MAP. Only at the two highest doses did effect deviate significantly from baseline; for some animals it was only the last dose that provided a significant deviation from slope of zero. For one animal, this response fell just short of that required to produce a negative linear regression test and therefore it was excluded.

3.2.2 Exclusion Criteria

The above describes the use of linear regression analysis to determine "zero effects" of drug-treatment or time on parameters of interest. This served a different function than the exclusion criteria, which were defined *a priori* and designed to eliminate animals with significant deviations in baseline response from later analysis. Normally these animals were replaced,

however in the group treated with RSD1020, one rat had to be excluded due to low mean arterial pressure at the start of the experiment but this was not apparent until subsequent analysis was performed, after the code for the experiment was broken, and so it was decided not to replace that animal. Therefore, all data for the RSD1020 group were calculated using four animals, rather than five.

3.2.3 Ventricular Stimulation Threshold

Potency for VFt (D25%) data is shown Table 6. Figure 6 shows the individual dose-response curves for each treatment group, including control data, for VFt. As noted above, there were no time-dependent effects on VFt observed in control animals. All drugs tested caused a dose-dependent increase in VFt.

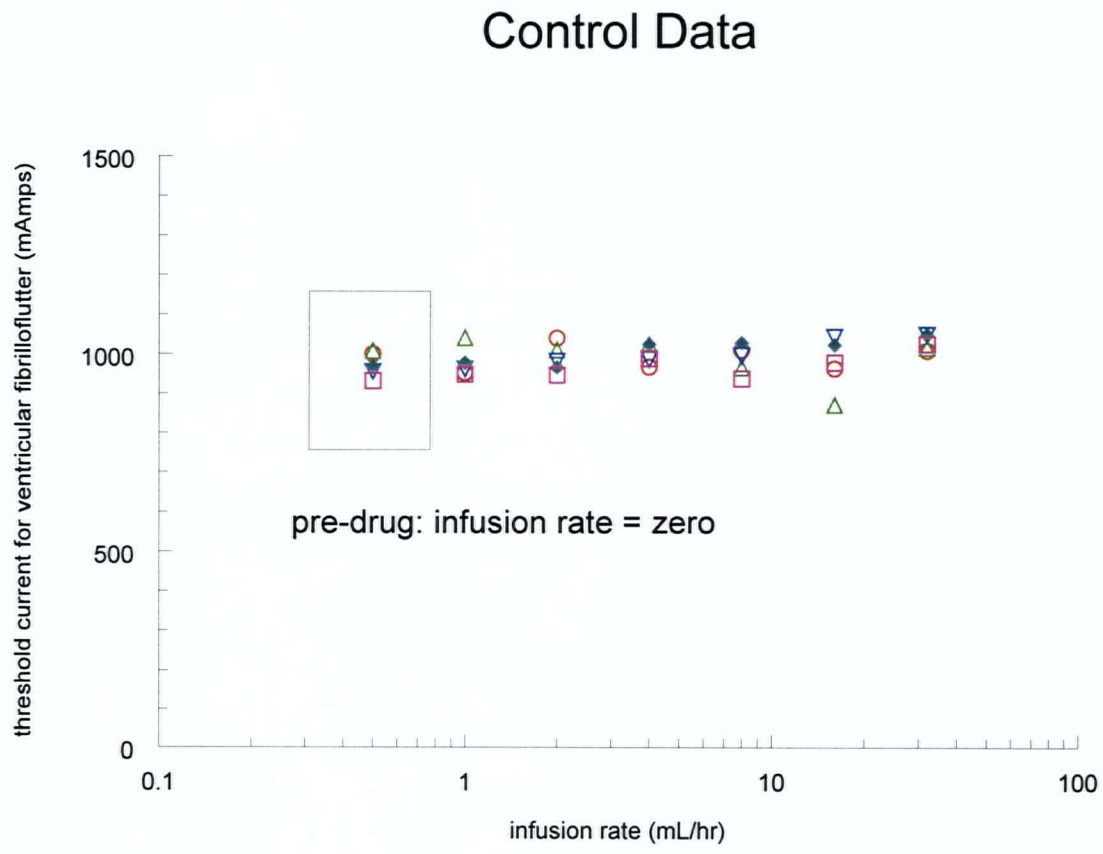
Table 6: Potency index for increase in ventricular stimulation threshold.

Drug	Mean D25% for increase in VFt (µmol/kg/min)	Upper error of the mean	Lower error of the mean
RSD1031	12.3	9.8	15.5
RSD1030	5.1	3.2	6.5
RSD1020	3.0	2.6	3.4
RSD995	1.3	0.9	1.8
RSD988	0.8	0.4	1.7
RSD944	0.3	0.3	0.4

Mean potency indices (D25%) and standard errors for effects on ventricular stimulation threshold (VFt), for each drug-treated group . D25% is the dose required to produce a 25% increase in VFt from pre-drug values. D25% was determined by extrapolation using dose-response curve equations for each animal. The D25% values for each animal in a treatment group were averaged using a method of geometric means, which yields errors that are not symmetrically distributed around the mean.

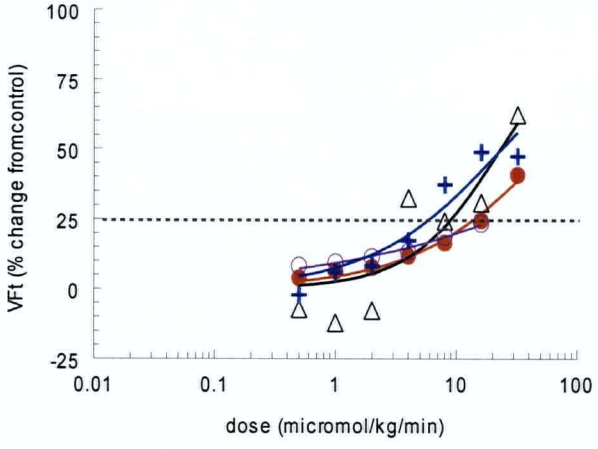
Figure 6: Control and drug-treated group dose-response curves for VFt from electrical stimulation experiments. A) raw VFt data (in mAmps) for control animals (infused with saline). "Pre-drug" phase is prior to start of infusion (i.e. infusion rate = 0). **B)** transformed VFt data for drug-treated groups. Ordinate shows percent change from control, calculated as described in Methods. Data for each animal in a treatment group were fit to a dose-response equation by non-linear regression (coloured curves). Dashed line shows 25% response level.

A

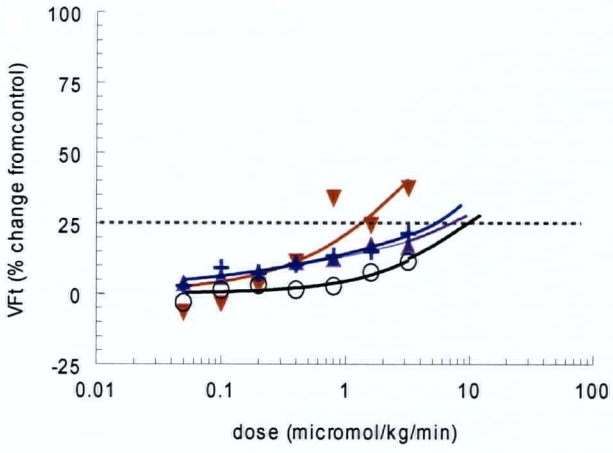


B

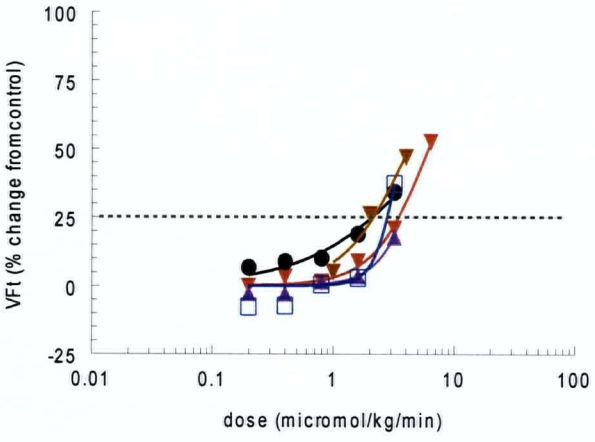
RSD1031



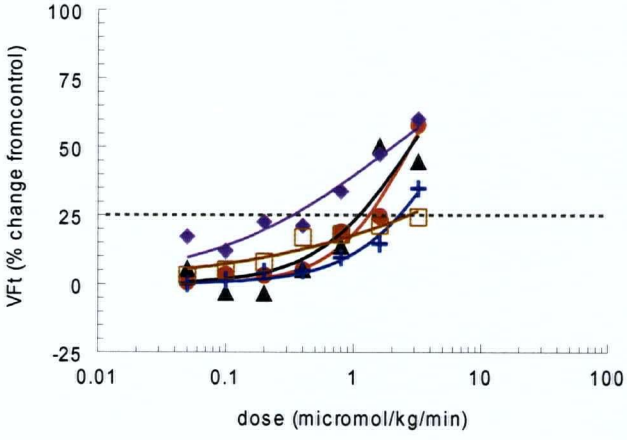
RSD1030



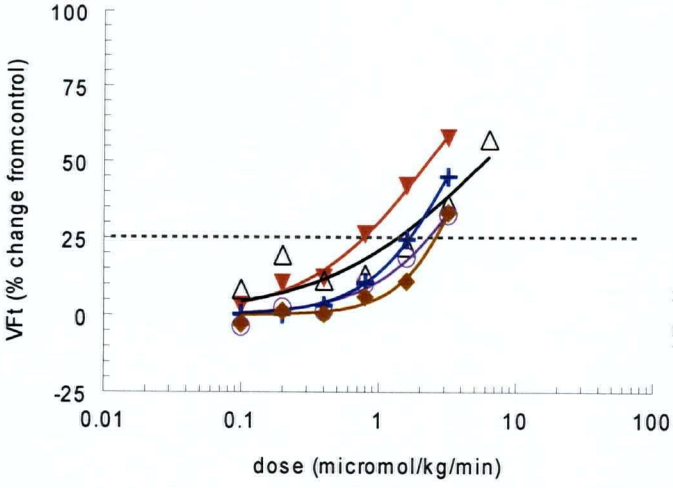
RSD1020



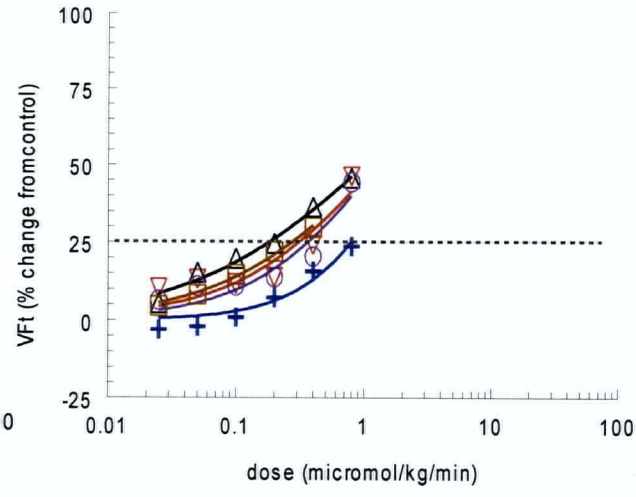
RSD995



RSD988



RSD944



3.2.4 Other Parameters

Potency indices (D25%) were also calculated for MAP, ERP, MFF, iT, PR, QT1, and QT2 for all treatment groups that passed the linear regression. Data are shown in Table 7.

RSD995, RSD988, and RSD944 were roughly equipotent for effects on excitability (iT, PR Interval) and repolarisation (ERP, MFF, QT1, and QT2). On the other hand, RSD1031, RSD1030, and RSD1020 were considerably more potent for effects on repolarisation than effects on excitability.

RSD995 had no discernable effect on blood pressure, and RSD1031 and RSD1030 had very little potency. On the other hand, RSD1020, RSD988, and RSD944 had significant effects on blood pressure over the range of doses tested.

3.2.5 Correlations

Correlation analysis between variables measured in these experiments show that measurement of ERP, MFF, QT1, and QT2 all correlated with one another. Thus the various indices of repolarisation effects gave consistent results. These indices also showed positive correlations with PR Interval effects. Thus, as effects on excitability increased so did effects on repolarisation. Effects on iT were not included in correlation analysis because for two drugs potency was not estimatible as response did not reach the 25% level.

Potency for effects on MFF showed a significant correlation with effects on VFt. This is illustrated in the following rankings for potency (most to least potent) for these two variables.

Potency indices for:

prolonging MFF:	**944 > 988 > 1020 > 995 > 1031 > 1030**
increasing VFt:	**944 > 988 > 995 > 1020 > 1030 > 1031**

Table 7: Potency indices for ECG and electrical stimulation variables.

Drug (RSD#)	D25% (upper SE, lower SE)						
	MAP	ERP	MFF	PR	iT	QT1	QT2
944	0.7 (0.9,0.6)	0.3 (0.5, 0.2)	0.3 (0.5, 0.2)	1.1 (1.1,0.8)	0.5 (0.6, 0.4)	0.4 (0.5, 0.4)	0.5 (0.7, 0.4)
988	2.0 (2.6, 1.5)	0.9 (1.1, 0.7)	0.5 (0.7, 0.3)	4.3 (4.9, 3.7)	2.8 (3.7, 1.5)	1.9 (2.3, 1.7)	1.0 (1.3, 0.8)
995	NE	2.0 (2.6, 1.6)	1.8 (2.5, 1.3)	2.5 (2.8, 2.3)	1.7 (2.9, 1.0)	NE	2.2 (2.2, 2.1)
1020	1.2 (1.6, 0.9)	0.8 (1.0, 0.7)	1.1 (1.3, 1.0)	10 (13, 7.6)	NE	0.9 (0.9, 0.8)	0.4 (0.4, 0.3)
1030	32 (40, 25)	8.7 (11, 7.2)	NE	56 (66, 48)	NE	6.0 (7.1, 5.3)	6.7 (8.0, 5.1)
1031	27 (32, 22)	6.3 (7.2, 5.5)	16 (19, 13)	41 (50, 33)	20 (23, 17)	4.8 (5.2, 4.4)	4.8 (5.1, 4.5)

Mean potency indices (D25%) and upper and lower standard error of the mean (in brackets) was calculated from dose-response curves for each animal in a group (n=5) using a method of geometric means. Errors are therefore not distributed equally about the mean. NE = not estimatible (ie. response did not reach 25% response level).

3.3 Selectivity for ischaemic versus non-ischaemic arrhythmias

Comparing results from coronary occlusion and electrical stimulation experiments yielded insight into the selectivity of the six test compounds for ischaemic versus non-ischaemic arrhythmias. The potencies for each could not be directly compared, since they were derived from different techniques (i.e. D25% versus ED_{50}). However, by comparing the rank order of potencies it is revealed that they are similar for both types of arrhythmia and results showed a strong correlation between the two ($r = 0.996$ vs. $r_{critical} = 0.811$, $p < 0.001$). The rank order of potency (from most to least potent) was as follows:

Protection against:	
ischaemia-induced arrhythmias:	**944* > 988 > 995 > 1020 > 1030 > 1031**
non-ischaemic arrhythmias:	**944 > 995 > 988 > 1020 > 1030 > 1031**

*944 could not be determined directly since it did not fit the regression equation. However, estimation of ED_{50} by eye suggests that it is the most potent of all compounds studied in terms of the dose that produced a half-maximum response (see Figure 5C).

Correlation analysis revealed an inverse relationship between potency for protection against both ischaemic (ED_{50}) non-ischaemic arrhythmias (VFt) and the goodness of fit (r^2) from coronary occlusion experiments. In other words, drugs which showed strong fits (least scatter about the curve) to dose-response curves for arrhythmia score (protection against ischaemia-induced arrhythmias) were least potent at protecting against both types of arrhythmia. The correlation coefficient was $r = -0.849$ and $r = -0.863$ for VFt and ED_{50}, respectively, vs. $r_{critical} = 0.811$, $p < 0.05$. This is illustrated in the following rankings of potency (strongest to least potent) and magnitude of r^2 (highest to lowest).

Protection against ischaemic arrhythmias:	**944* > 988 > 995 > 1020 > 1030 > 1031**
Protection against non-ischaemic arrhythmias:	**944 > 995 > 988 > 1020 > 1030 > 1031**
r^2 for ischaemic arrhythmia dose-response curves:	**1031 > 1020 > 1030 > 995 > 988 > 944**

*944 could not be determined directly since it did not fit the regression equation. However, estimation of ED_{50} by eye suggests that it is the most potent of all compounds studied in terms of the dose that produced a half-maximum response (see Figure 5C).

Another significant correlation was found between potency for decreasing MFF in normal tissue and potency for protection against ischaemia-induced arrhythmias: $r_S = 0.970$ vs. $r_{S,critical} = 0.886$, $p < 0.05$. In other words, drugs which were most potent at reducing maximum following frequency (i.e. increasing the refractory period) in normal tissue were also most potent at protecting against ischaemia-induced arrhythmias. This is illustrated in the following rank orders of potency:

protection against ischaemic arrhythmias: **944* > 995 > 988 > 1020 > 1030 > 1031**
decreasing maximum following frequency: **944 > 995 > 988 > 1020 > 1030 > 1031**

*944 could not be determined directly since it did not fit the regression equation. However, estimation of ED_{50} by eye suggests that it is the most potent of all compounds studied in terms of the dose that produced a half-maximum response (see Figure 5C).

3.4 Isolated Heart Experiments

3.4.1 Optical Action Potentials

3.4.1.1 Signal Fidelity

Pilot experiments showed a time-dependent decrease in maximum rise rate in both recording areas (no occlusion performed). Results from a representative pilot experiment are shown in Figure 7. This decrease could be attributed to dye washout since baseline values could be restored by adding another dose of dye, consistent with the results reported by Windisch et al [162].

The duration of exposure to excitation light also affected signal fidelity. In two pilot experiments, measurements of maximum upstroke velocity were made using intermittent acquisitions (i.e. excitation lamps were turned on only during the acquisition period, which lasted approximately 3-5 seconds), or continuous acquisition (i.e. lamps were on for the duration of the experiment). The results showed a photobleaching effect where exposure to excitation light caused an apparent time-dependent decrease in maximum upstroke velocity. The results are shown in Figure 8.

Figure 7. Pilot experiment showing effect of dye washout on maximum upstroke velocity of optically detected cardiac action potentials. This experiment was performed as described in Methods for sham occlusion experiments. The "ischaemic zone" corresponds to the area that would be occluded had the occluder been tightened. Data was recorded at the times indicated on the abscissa. The dose of dye was 20 μL of a 2 mM stock solution (slow bolus injection into perfusate stream), as described in Methods, and was re-administered at the time shown by the arrow. The graph shows that the apparent decrease in maximum upstroke velocity with time could be accounted for by dye washout. Maximum upstroke velocity is shown in AD units per second, which is directly proportional to V/sec.

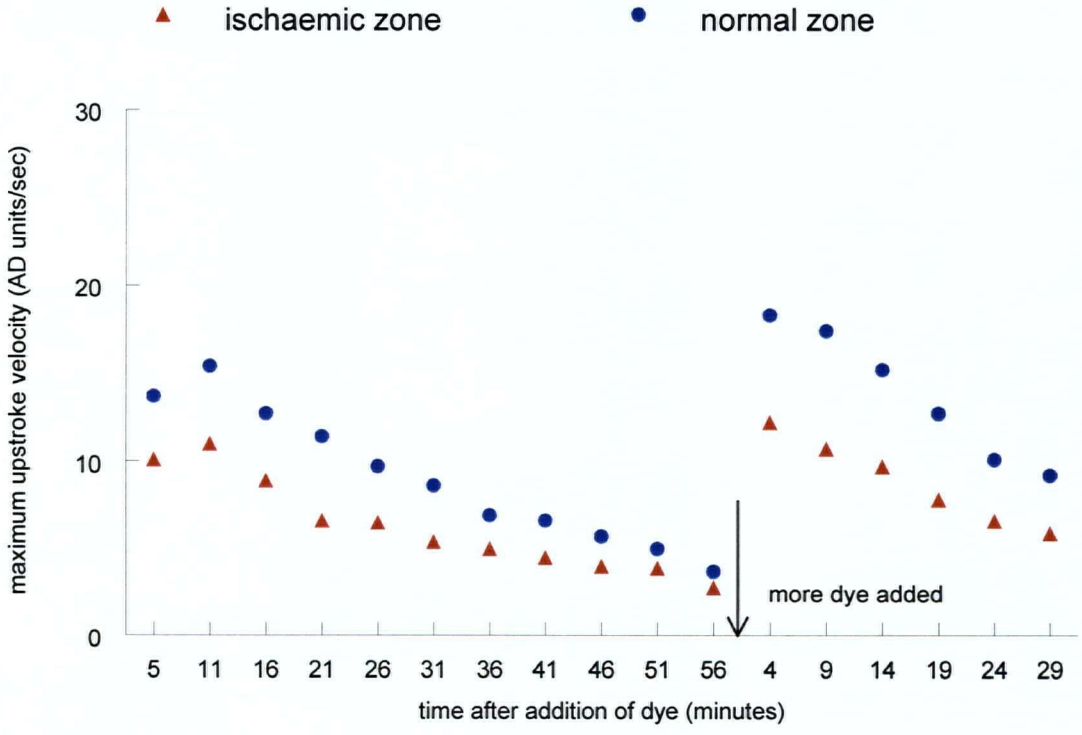
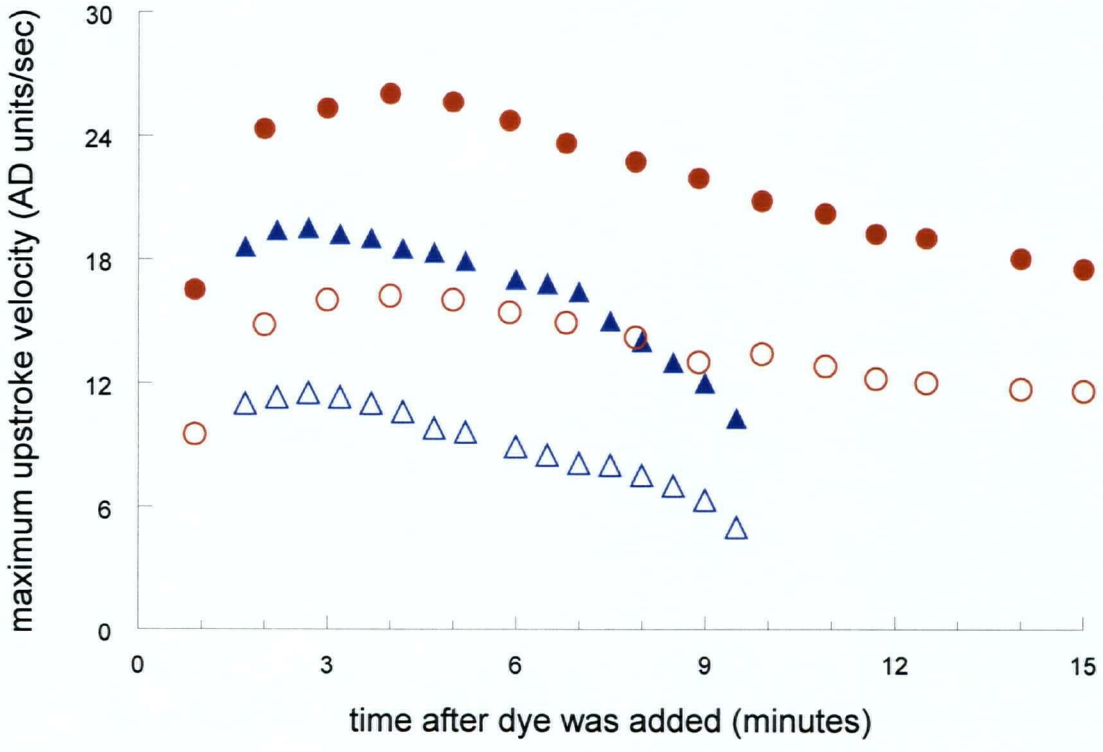


Figure 8. Pilot experiment showing photobleaching effect on dye. Experiments were performed as described in Methods for sham-occlusion experiments. In one protocol (blue triangles) excitation lamps were left on for the duration of the experiment and maximum upstroke velocity was measured at various times throughout the single recording. In another protocol (red circles) recordings were made only at the desired time points so that excitation lamps were on only during the acquisition, which lasted 3–5 seconds. When lamps were left on continuously, maximum upstroke velocity decreased significantly in both recording areas (normal zone = open symbols, sham-ischaemic zone = filled symbols).



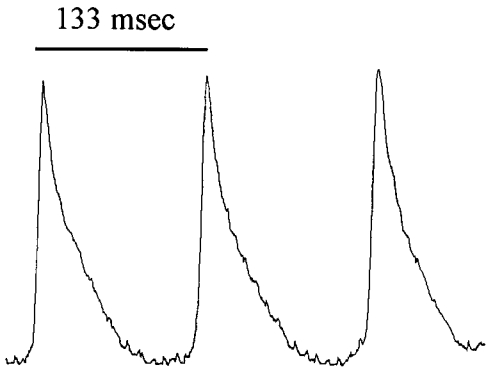
The signal-to-noise ratio (SNR) for these signals was measured using standard techniques [153], with a minimum acceptable SNR of 40, as described in Methods (Section 5.3.6). SNR was greater than 40 for all experiments until some time between 4 and 5 minutes following occlusion. Action potentials were measured at three minutes post-occlusion when SNR was still greater than 40 in all experiments. Figure 9 shows representative optical action potentials from a number of experiments. For the pre-occlusion signal shown in the top right, SNR was 75; at three minutes post occlusion in the ischaemic zone (middle right) it was 69. In a representative signal from a drug-treated heart (bottom right) SNR in ischaemic zone recordings after three minutes occlusion was 57. There was some evidence of 60 Hz hum in these signals, but fortunately the magnitude was not enough to interfere with resolution of action potentials at the points of interest (i.e. SNR was still > 40).

3.4.1.2 General Experimental Effects on Optical Action Potentials

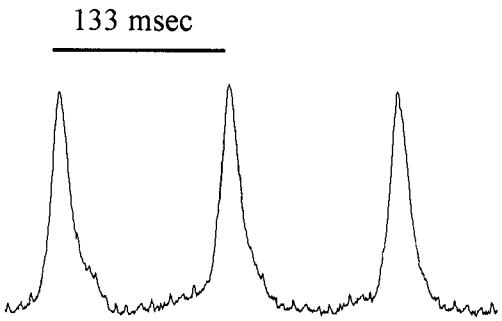
Figure 9 shows representative action potentials from before and after occlusion. In general, the effect of drug was to decrease V_{max} and prevent ischaemia-induced shortening of action potential duration. It was not possible to calculate absolute maximum upstroke velocity in this experiment due to the nature of optical action potentials (see Introduction section 5.2.3). However, measurements of APD agree with those reported by other investigators. For example, Spear used intracellular microelectrodes to record action potentials from superfused strips of rat ventricular myocardium and reported APD_{50} of 25.6 ± 2.4 msec. Control APD_{50} values in the present study ranged from 15-25 msec. This may be explained by the fact that the density of I_{to}, the primary repolarising current in rat cardiac action potentials, is much greater in epicardial cells than endocardium and APD_{50} is markedly shorter in epicardial cells because of this fact [163]. In this experiment recordings were made from the epicardial layer, as excitation light does not penetrate beyond a few cell layers [153].

Figure 9: Examples of optical action potentials. Signals were recorded from perfused (right column) and occluded (left column) regions. In all experiments hearts were paced at 133 msec cycle length. Top row shows typical pre-occlusion action potentials, shown here from a control experiment. Also shown are action potentials at three minutes after occlusion of the left coronary artery in other control hearts (middle row) and hearts treated with 3.0 μM RSD1030 (bottom row). The y-axis is a unitless AD (analog-to-digital conversion constant) value that represents change in fluoresence relative to background (ΔF/F), which is directly proportional to membrane voltage. Thus, action potential amplitude cannot be directly compared between experiments and the y-axis is not shown here. The x-axis is time in msec; scale bars are shown for each signal. The presence of 60 Hz hum was seen in all signals, but this did not obscure measurements of upstroke velocity or action potential duration.

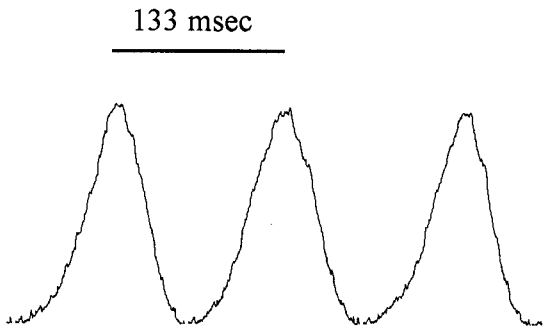
ISCHAEMIC ZONE



Pre-occlusion: control

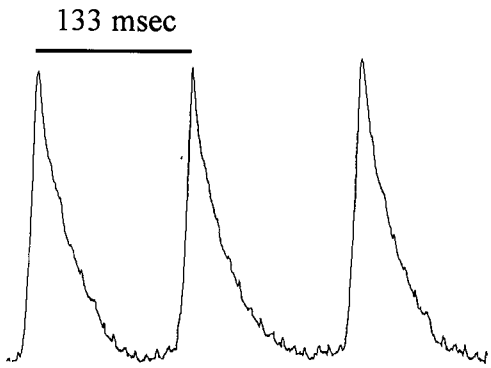


3' post-occlusion: control

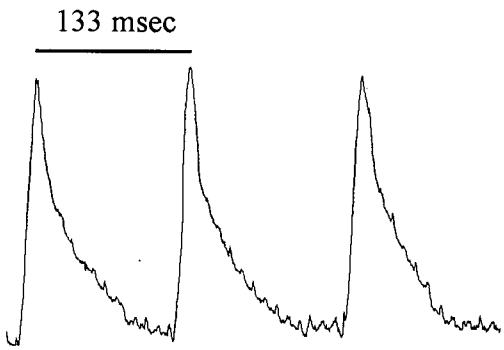


3' post-occlusion: 3.0 μM RSD1030

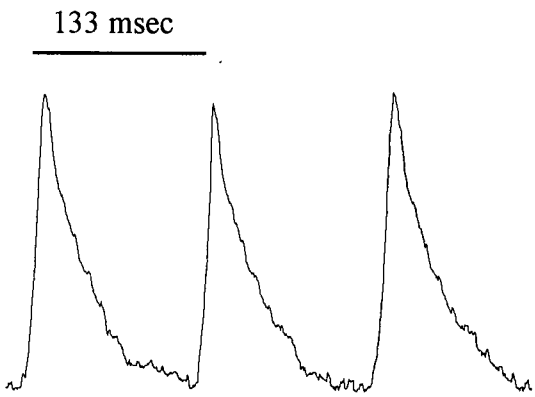
NORMAL ZONE



Pre-occlusion: control



3'post-occlusion: control



3' post-occlusion: 3.0 μM RSD1030

Time control experiments in drug-free occluded hearts are shown in Figure 10. A slight apparent decrease in V_{max} was observed in the normal zone which, as described above, could be attributed to dye washout. The effect of ischaemia was to significantly slow V_{max} in a time-dependent manner beyond the level attributable to dye washout. APD did not change over time in the normal zone, but in the ischaemic zone there was a time-dependent decrease in APD_{50}, APD_{75}, and APD_{90}, while APD_{25} showed a slight increase.

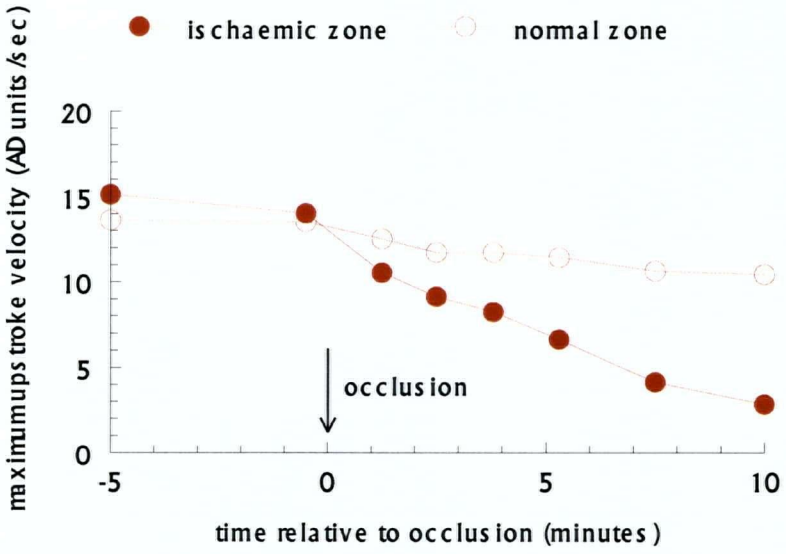
3.4.2 Control and Sham-Occluded Groups

Two sets of control groups were used: occluded and sham-occluded. Both groups were prepared according to the protocol outlined in Methods. Data for percent change in V_{max} is shown in Figure 11 (see Appendix C for raw data). There was no significant difference in ΔV_{max} between normal and sham-occluded regions in sham-treated hearts (24.0 ± 2.7% and 23.0 ± 4.4%, mean ± S.E.M., respectively). There was also no significant difference in ΔV_{max} between either region of sham-treated hearts and the normal region of occluded control hearts, which was 23.2 ± 2.5%. However, in occluded controls ischaemia caused a decrease in V_{max} such that ΔV_{max} in the ischaemic zone was significantly greater than in the normal zone (55.7 ± 2.8%, $p < 0.0005$).

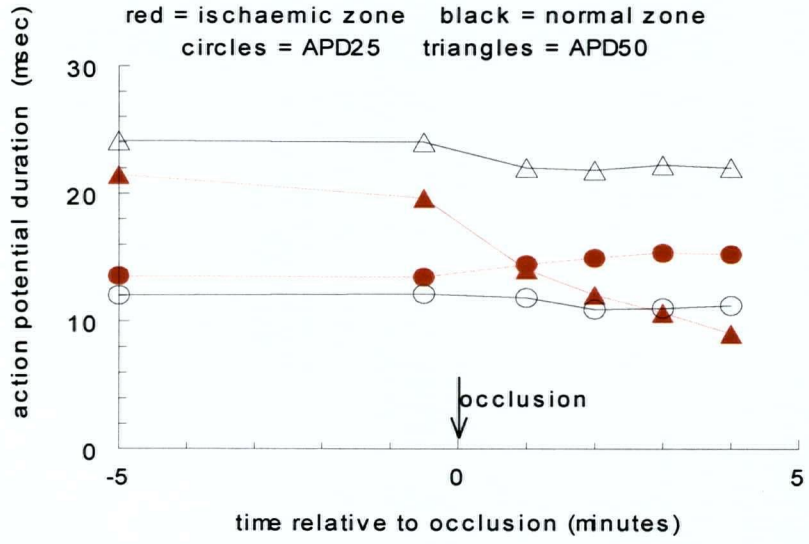
Percent change in action potential duration at 25, 50, 75, and 90% repolarisation (ΔAPD) was determined for occluded and sham-occluded controls. Data are shown in Figure 12. In sham-occluded hearts there was no significant difference in ΔAPD between normal and sham-occluded regions, but in control hearts ΔAPD was different in the occluded zone compared to the normal zone. Note that APD_{25} was increased by ischaemia, whereas APD_{50}, APD_{75}, and APD_{90} were decreased by ischaemia.

Figure 10. Time course of changes in maximum upstroke velocity and action potential duration in normal and ischaemic zones following occlusion. This data was taken from a pilot time-control experiment using a drug-free occluded heart. **A.** upstroke velocity in the normal zone (open circles) and ischaemic zone (filled circles) at baseline (-5 min), pre-occlusion (-1 min), and at various times after occlusion. **B.** APD_{25} (circles) and APD_{50} (triangles) in the normal zone (open symbols) and ischaemic zone (filled symbols) at various times before and after occlusion (occlusion at t=0 minutes). **C.** APD_{75} (squares) and APD_{90} (diamonds) in the normal zone (open symbols) and ischaemic zone (filled symbols) at various times before and after occlusion (occlusion at t=0 minutes).

A


ischaemic zone
normal zone
maximumupstroke velocity (AD units/sec)
20
15
10
5
0
occlusion
-5
0
5
10
time relative to occlusion (minutes)

B


red = ischaemic zone
black = normal zone
circles = APD25
triangles = APD50
action potential duration (msec)
30
20
10
0
occlusion
-5
0
5
time relative to occlusion (minutes)

C

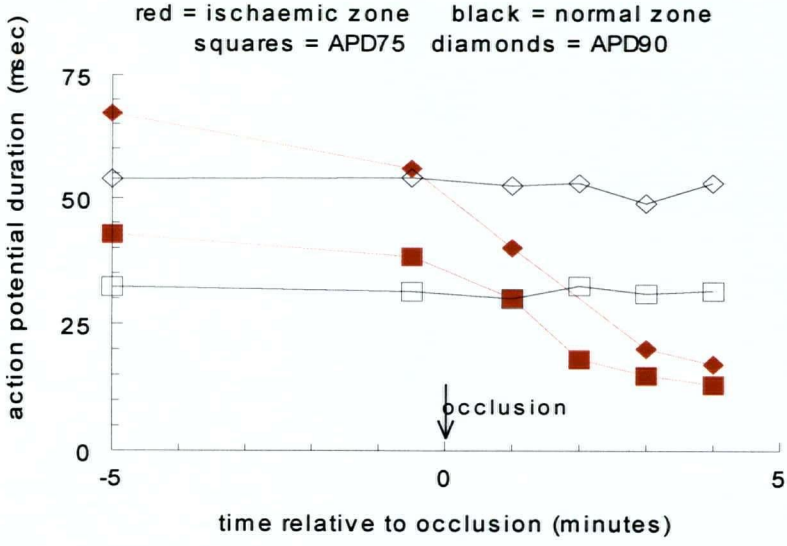

red = ischaemic zone
black = normal zone
squares = APD75
diamonds = APD90
action potential duration (msec)
75
50
25
0
occlusion
-5
0
5
time relative to occlusion (minutes)

Figure 11. Percent change in V_{max} for control experiments. Control experiments were performed as described in Methods. Two groups were used: occluded (right) and sham-occluded (left). Bars are mean ± S.E.M. Percent change in maximum upstroke velocity (ΔV_{max}) is shown for normal (red bars) and ischaemic (or "sham ischaemic") (blue bars) regions. * significantly greater than normal zone ($p < 0.05$)

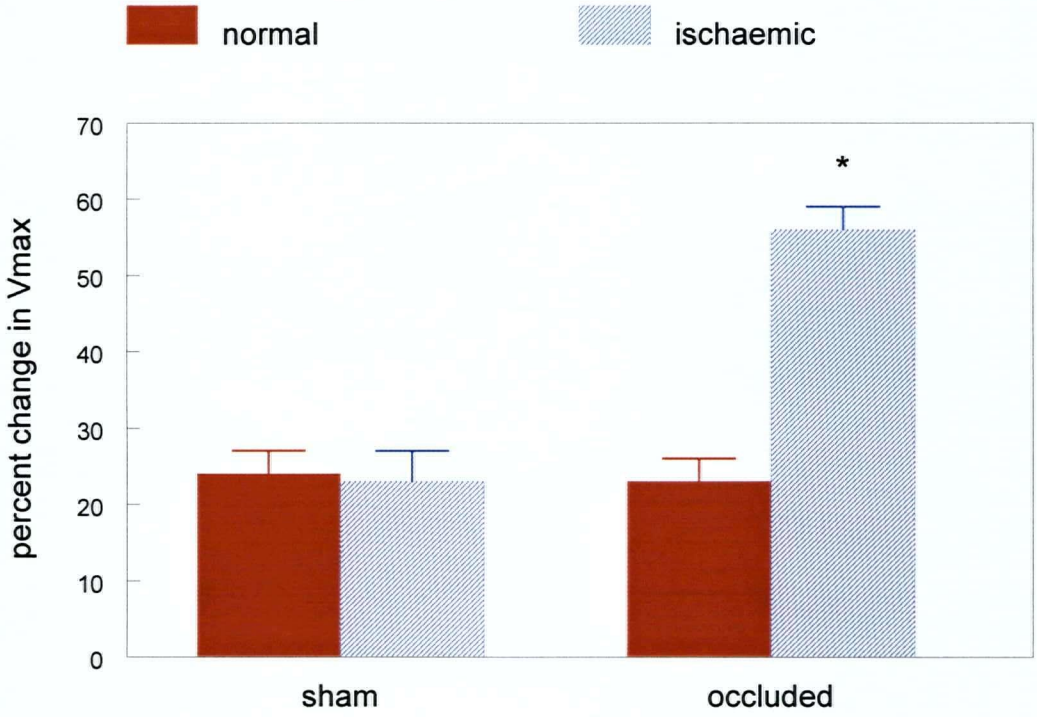
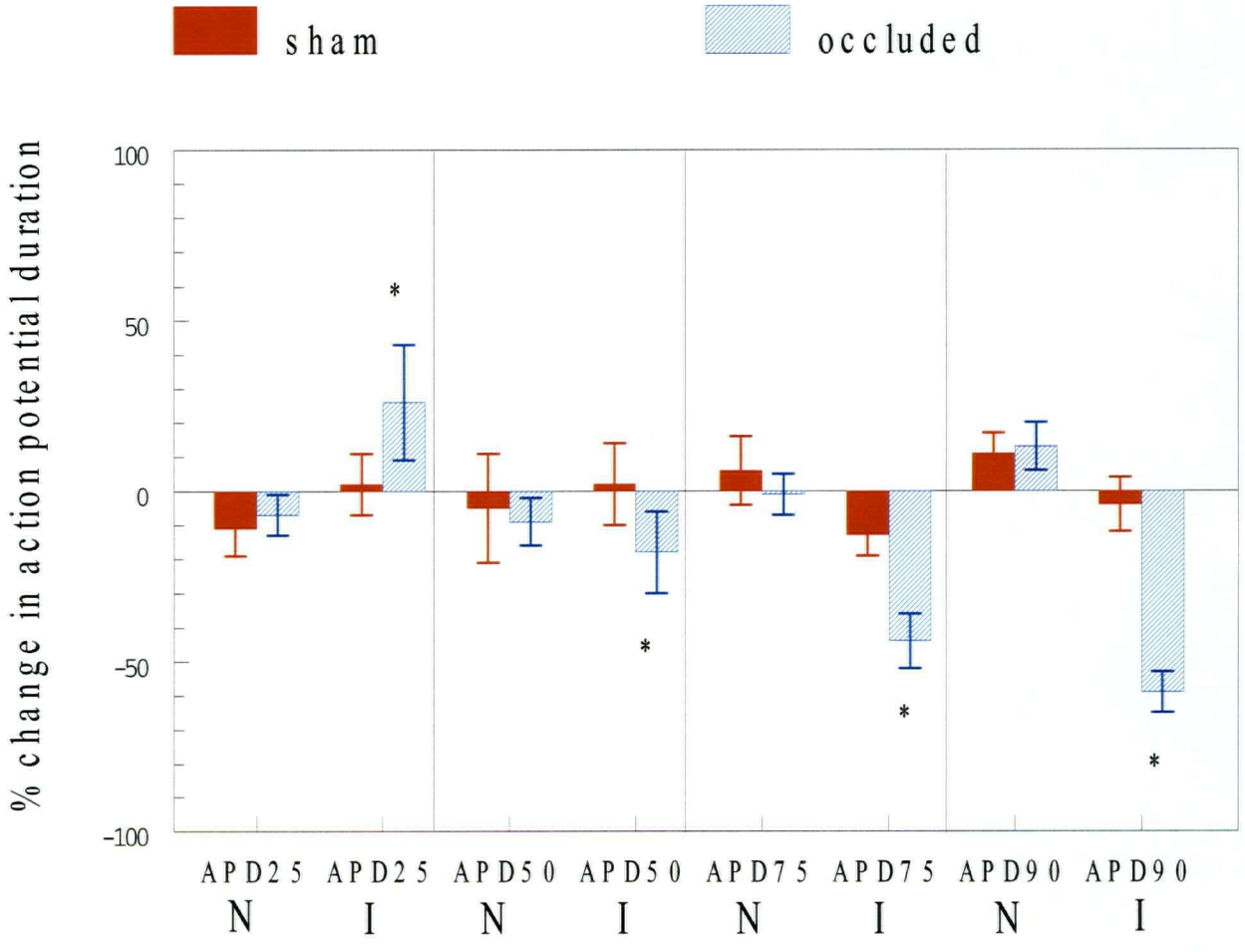


Figure 12. Percent change in APD for control experiments. Control experiments were performed as described in Methods. Two groups were used: occluded (blue hatched bars) and sham-occluded (red bars). Percent change in action potential duration (ΔAPD_{25}, ΔAPD_{50}, ΔAPD_{75}, and ΔAPD_{90}) are shown for normal (N) and ischaemic (or "sham-ischaemic") (I) regions. Percent change was calculated at three minutes post-occlusion relative to start of experiment. Bars are mean ± S.E.M. * Significantly different from normal zone ($p < 0.05$)



3.4.3 Drug-treated groups

Two doses of each test compound were used to examine drug effects on ΔV_{max} and ΔAPD in occluded hearts. Data for ΔV_{max} are shown in Figure 13. Data for ΔAPD is shown in Table 8. Data for ΔAPD_{50}, which was used for ISI calculations, are shown in Figure 14.

With respect to conduction effects, RSD1031 and RSD1030 had no effect on ΔV_{max} at either dose tested in the normal zone. However, RSD1030 significantly decreased conduction in the ischaemic zone, relative to the effects of ischaemia alone. RSD1020, RSD988, and RSD944 all decreased conduction at both doses tested, in both the normal and the ischaemic zone. RSD995 had no effect in either zone at the low dose, but did signficantly decrease V_{max} in both zones at the high dose.

With respect to effects on APD_{50}, which was used to calculate ischaemia-selectivity indices (see below), all drugs caused a significant increase in APD_{50} in both the normal and ischaemic zones at the highest dose tested, except for RSD1031 which had no significant effect in the normal zone at the highest dose. All drugs except RSD988 caused a signficant increase in APD_{50} in the ischaemic zone, but not the normal zone, at the lowest dose tested. RSD988 caused a significant increase in APD_{50} at the lowest dose in the normal zone, but did not affect APD_{50} in the ischaemic zone at the same dose. As can be seen in Table 8, the effects of drug on APD_{25} and APD_{50} tended to be similar, and greater than effects on APD_{75} and APD_{90}.

Figure 13. Percent change in V_{max} for drug experiments. Bars are mean ± S.E.M. Data shows percent change in maximum upstroke velocity (ΔV_{max}) for normal zone (red bars) and ischaemic zone (blue bars) at low and high doses (shown on abscissa). Control data are shown for comparison for normal zone (red horizontal line) and ischaemic zone (blue dashed horizontal line). **A.** Data for RSD1031, RSD1030, and RSD1020. **B.** Data for RSD995, RSD988, RSD944. *significantly different from control ($p < 0.05$)

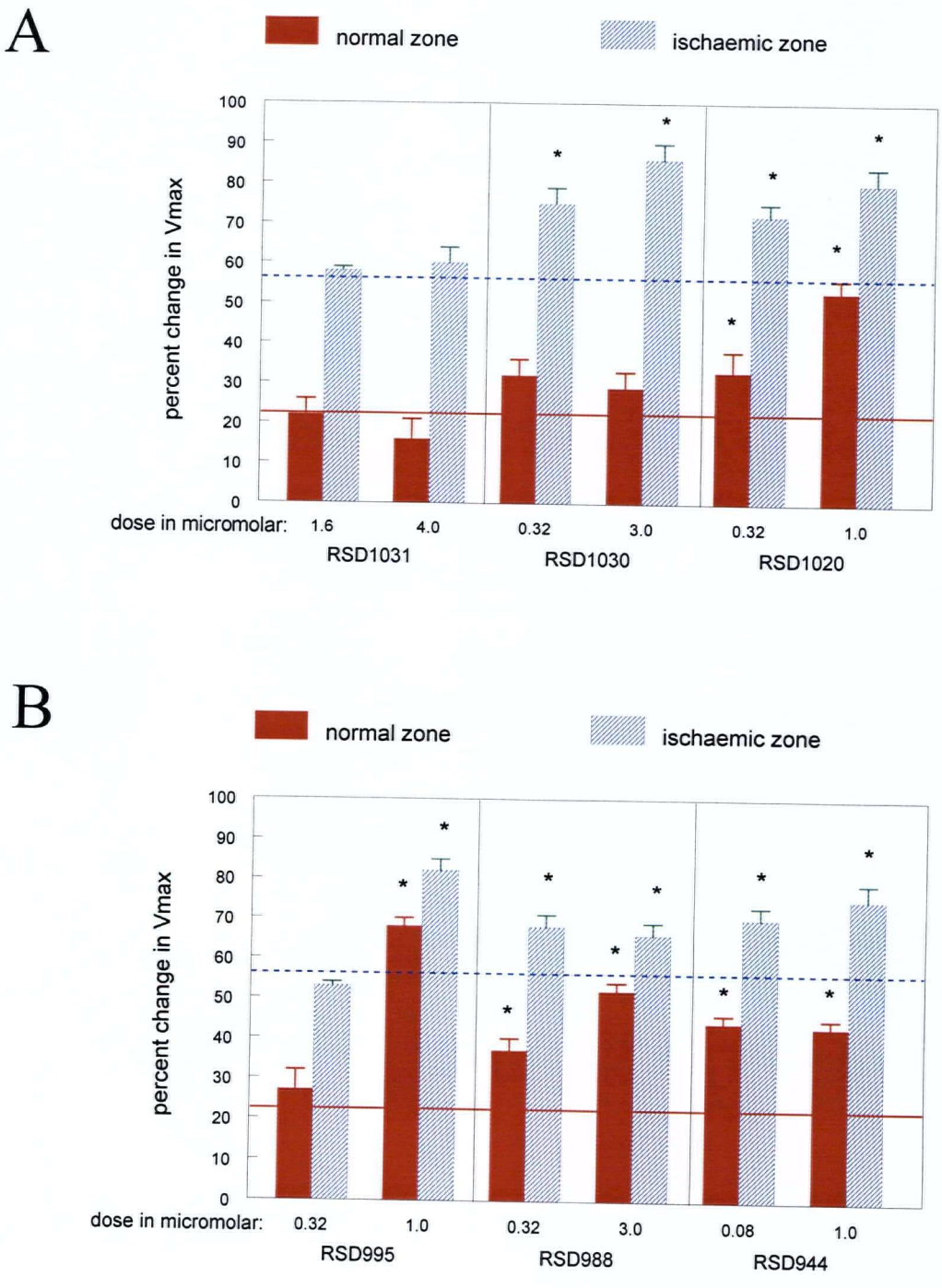


Table 8: Mean change in action potential duration in drug-treated hearts.

a) low dose

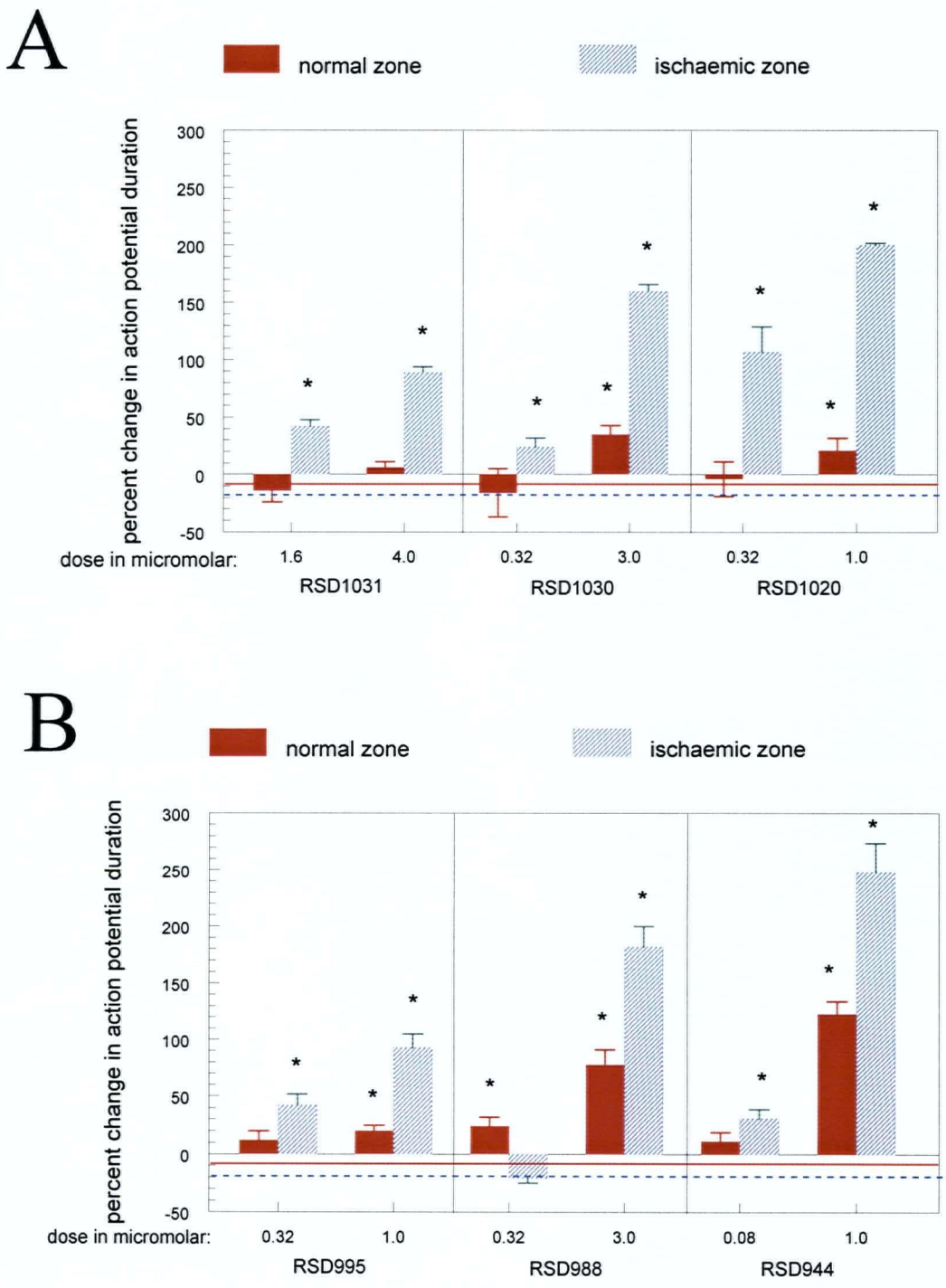
Drug Dose (μM)	Normal				Occluded			
	APD_{25}	APD_{50}	APD_{75}	APD_{90}	APD_{25}	APD_{50}	APD_{75}	APD_{90}
RSD944 (0.08)	15 ± 7	11± 8	8 ± 4	18 ± 7	52 ± 13	31 ± 8	4 ± 7	-6 ± 7
RSD988 (0.32)	21 ± 7	24 ± 8	44 ± 5	72 ± 13	34 ± 17	-21 ± 4	-39 ± 5	-56 ± 6
RSD995 (0.32)	13 ± 9	12 ± 8	22 ± 9	26 ± 12	54 ± 11	42 ± 10	32 ± 10	25 ± 6
RSD1020 (0.32)	10 ± 13	-4 ± 15	15 ± 22	53 ± 18	180 ± 21	107 ± 22	39 ± 24	16 ± 23
RSD1030 (0.32)	-9 ± 5	-16 ± 21	-10 ± 31	6 ± 29	55 ± 6	24 ± 8	-22 ± 7	-40 ± 10
RSD1031 (1.6)	-2 ± 7	-14 ± 10	-8 ± 14	14 ± 14	43 ± 8	42 ± 6	25 ± 8	12 ± 7

b) high dose

Drug Dose (μM)	Normal				Occluded			
	APD_{25}	APD_{50}	APD_{75}	APD_{90}	APD_{25}	APD_{50}	APD_{75}	APD_{90}
RSD944 (1.0)	158 ± 9	123 ± 11	66 ± 11	23 (n=2)	309 ± 28	248 ± 26	184 ± 21	NE
RSD988 (3.0)	182 ± 8	78 ± 13	45 ± 21	34 ± 36	199 ± 14	182 ± 18	142 ± 14	163 ± 9
RSD995 (1.0)	47 ± 3	20 ± 5	40 ± 5	34 ± 9	98 ± 13	93 ± 12	41 ± 11	23 ± 8
RSD1020 (1.0)	58 ± 9	21 ± 11	23 ± 9	9 ± 13	230 ± 7	200 ± 0	154 ± 11	133 ± 17
RSD1030 (3.0)	48 ± 6	35 ± 8	28 ± 11	18 ± 1	225 ± 15	160 ± 6	112 ± 16	65 ± 14
RSD1031 (4.0)	6 ± 6	6 ± 5	14 ± 8	13 ± 5	113 ± 7	89 ± 5	45 ± 4	32 ± 7

Change in action potential duration (ΔAPD; mean ± S.E.M.) in hearts treated with one of six test compounds at low (a) and high (b) doses (shown in brackets) in normal and ischaemic (occluded) zones. NE = not estimatible. These values were used to calculate ISR-D for each drug.

Figure 14. Percent change in APD_{50} for each of six drug-treated groups. The percent change in action potential duration at 50% repolarisation (ΔAPD_{50}) is shown for low and high doses (shown on abscissa in micromolar) for normal zone (red bars) and ischaemic zone (blue bars). Control experiment data are shown for comparison for normal zone (red horizontal line) and ischaemic zone (blue dashed horizontal line). **A.** Data for RSD1031, RSD1030, and RSD1020. **B.** Data for RSD995, RSD988, RSD944. * significantly different from control values ($p < 0.05$)



3.4.4 Ischaemia-Selectivity of Test Compounds

To determine whether ischaemia potentiated the effects of drug on V_{max} and APD, ischaemia-selectivity indices (ISI-V and ISI-R) were calculated using various techniques as described in Methods. These results are shown in Tables 9-11. For one compound, RSD1031, effects on V_{max} in normal and ischaemic tissue were not significantly different than controls even at the highest concentration, therefore ISI-V was not determined for this compound.

With respect to effects on V_{max} the orders of selectivity for ischaemic tissue as determined by the three methods are:

Response Ratio:	**1030 > 944 > 1020 > 995 > 988**
Parallel Shift:	**1030 > 1020 > 995 > 988 > 944**
Potency Shift:	**1030 > 944 > 988 > 1020 > 995**

With respect to effects on APD_{50}, the orders of selectivity for ischaemic tissue as determined by the three methods are:

Response Ratio:	**1020 > 1031 > 1030 > 995 > 988 > 944**
Parallel Shift:	**1020 > 995 > 1030 > 1031 > 944 > 988**
Potency Shift:	**1020 > 1030 = 944 > 988 > 1031 > 995**

The Response Ratio values were determined to most closely represent the data shown in Figures 13 and 14 (the rationale for this is presented below in the Discussion). Therefore, ISI values from this method were used in correlation analysis. Correlation analysis revealed a positive relationship between ISI-R and both slope and goodness of fit (r^2) from coronary occlusion experiments ($r = 0.884$ and 0.886, respectively, versus $r_{critical} = 0.811$, $p < 0.05$). Thus, compounds which were most selective for ischaemic tissue had the highest r^2 values and the steepest slopes for antiarrhythmic dose-response curves.

Table 9: Ischaemia-selectivity indices determined using the Response Ratio method.

Compound	ISI-V	ISI-R
RSD944	0.95	2.02
RSD988	0.34	2.30
RSD995	0.58	3.83
RSD1020	0.80	7.27
RSD1030	5.00	4.05
RSD1031	NE	7.13

ΔV_{max} and ΔAPD_{50} values for control hearts were subtracted from those of drug-treated hearts to account for effects of time and ischaemia. The resulting values were divided (ischaemic:normal) to yield an index of ischaemia-selectivity, ISI. An ISI value greater than one indicates that drug effects in ischaemic tissue were greater than in normal tissue. An ISI value less than one indicates drug effects in normal tissue were greater than effects in ischaemic tissue. ISI-V is ischaemia-selectivity index for effects on V_{max}. ISI-R is ischaemia-selectivity index for effects on APD_{50}. NE = could not be determined

Table 10: Ischaemia selectivity indices determined using the Parallel Shift method

Compound	ISR-V	ISR-R
RSD944	-2.19	0.54
RSD988	-1.57	0.29
RSD995	-0.18	1.01
RSD1020	0	1.35
RSD1030	4.13	0.95
RSD1031	NE	0.93

ISR values calculated using Parallel Shift method. A 2 x 2 bioassay potency shift equation was used to determine ischaemia-selectivity for effects on V_{max} and APD_{50}, as described in Methods. The formula assumes equal slopes for both normal and ischaemic dose-response curves. Positive ISR values indicate that drug was more potent in ischaemic tissue than in normal tissue. Negative values indicate that drug was more potent in normal tissue than in ischaemic tissue. A value of zero indicates equal potency in both normal and ischaemic tissue. NE = could not be determined.

Table 11: Ischaemia selectivity indices determined using the Potency Shift method

Compound	ISR-V	ISR-D
RSD944	-1.10	10.1
RSD988	-1.20	7.00
RSD995	-8.90	4.10
RSD1020	-1.80	25.5
RSD1030	17.3	10.1
RSD1031	NE	4.80

ISR values calculated using Potency Shift technique, as described in Methods. Mean slope was determined by calculating slope for all treatment groups where both doses appeared to fall on the linear portion of the dose-response curve (i.e. both doses produced responses that were significantly different from controls). The mean slope was then used to determine potency shift by calculating the ratio of doses at a given response level. A positive value indicates that drug was more potent in ischaemic tissue than in normal tissue. A negative value indicates that drug was more potent in normal tissue than in ischaemic tissue. NE = could not be determined.

ISI-V was shown to be inversely correlated with D25% for effects on PR Interval, ERP, QT1, QT2, and VFt from electrical stimulation experiments ($r = 0.983, 0.967, 0.933, 0.940, 0.856$ respectively, versus $r_{critical} = 0.811$, $p < 0.05$). Examination of ISI-V values in Table 9 show that for four out of five compounds effects on excitability were selective for normal over ischaemic tissue (values less than one). Thus the positive correlations above show that drugs which were more selective for normal tissue were more potent at suppressing non-ischaemic arrhythmias, and had more potent effects on excitability and repolarisation in normal tissue.

3.5 Correlation Analysis

The results of correlation analysis are summarised below. Table 12 shows significant positive and negative (inverse) correlations for comparisons of potency indices from coronary occlusion and electrical stimulation experiments (see Table 3 for list of variables) and ISI values determined using the Response Ratio method.

Table 12: Summary of significant correlation relationships.

Significant Correlations
MFF α ERP, QT1, QT2, PR, VFt, ED_{50}
ERP α QT1, QT2, PR, ISI-V
QT1 α QT2, PR, ISI-V, MAP
QT2 α PR, ISI-V
VFt α ED_{50}, ISI-V, $1/r^2$
PR α ISI-V
ED_{50} α $1/r^2$
ISI-R α r^2, slope

Significant ($p < 0.05$) positive and negative (inverse) correlation relationships as determined by correlation analysis. ISI values were calculated using the Response Ratio method. For definition of variables see Table 3.

CHAPTER 4: DISCUSSION

The electrophysiologic changes that occur in myocardial tissue during an ischaemic episode have been well documented [164]. The increased incidence of arrhythmia that coincides with acute ischaemia is almost certainly due to the functional changes occurring in the ischaemic region, while tissue in the perfused zone behaves relatively normally with respect to impulse propagation and repolarisation [52]. Thus it has been suggested that drugs which act in ischaemic tissue may prove to be more effective at suppressing ischaemia-induced arrhythmias than drugs which affect normal electrophysiologic function in the perfused zone, and/or lose their effectiveness under conditions of ischaemia. This thesis is a continuation of previous work in the laboratory that supports this hypothesis (see section 1.4).

This section will begin with a discussion of the suitability of the methods used to investigate the central hypothesis of this thesis, based on the results and those of other investigators. This will include the use of optical action potentials to study electrophysiologic effects of drugs and the effect of ischaemia on drug action. Following this, results will be interpreted with respect to the central hypothesis, and the limitations and future directions of this study will be discussed.

4.1 Use of the Rat as an Experimental Model

Numerous species of experimental animal have been used in cardiac research [165] yet no one species has been shown to act as an ideal model for human cardiac physiology and pathophysiology. Small animals are generally preferable to large ones due to the expense of purchasing animals and the extensive housing and surgical facilities required for larger animals. Where the supply of experimental drug is limited, smaller animals offer the added advantage of requiring less drug. The rat was chosen for this thesis because the laboratory has extensive

experience with various rat models of cardiac arrhythmia, and because the experimental drugs used in this study were limited in supply, and had been previously characterised in rats.

The disadvantage of this model is the fact that rat ventricular action potentials more closely resemble human atrial, rather than ventricular action potentials. In rats, repolarisation is primarily driven by the transient outward potassium current, I_{to} whereas in other mammalian ventricles I_{Kr} and I_{Ks} are the predominant currents [163]. Interestingly, I_{to} is found to be most dense in human [166], rat [167], and mouse [168] while its density is very low in guinea pigs [169]. Certain pathophysiological conditions that involve I_{to} may therefore be better modelled in rats and mice [163], but in normal tissue at least there are considerable differences in repolarising currents between species, albeit well-defined ones.

On the other hand rats and humans do not possess extensive coronary collaterals whereas dogs and guinea pigs do [170]. This has significant effects on the outcome of coronary occlusion in terms of size of the occluded zone and the nature of the border region. Bergey et al [24] showed that while rats exhibited uniform ventricular fibrillation during coronary occlusion, more than 30% of dogs survived the procedure. Because the size of the ischaemic zone is directly related to the incidence and severity of arrhythmias, it was important in this study to use coronary occlusion models where the size of the ischaemic region was reproducible and could be precisely controlled. Since the major focus of this study was the effect of ischaemia on drug action this was of primary consideration when choosing an experimental species.

4.2 Coronary Occlusion Experiments

A common pathological cause of arrhythmia in humans is the occurrence of myocardial ischaemia either alone, progressing to infarction, or with reperfusion. These events can be readily reproduced in intact rats [24]. Coronary occlusion in the rat has been used extensively to study

the antiarrhythmic effects of various drugs [25;171;172]. For reviews see Curtis and Walker [160], or Cheung et al [173].

The incidence and severity of arrhythmias observed in the present study correlated well with those reported elsewhere [174], as did the mean arrhythmia score for control animals [159]. ED_{50} values for RSD944, RSD988, RSD995 and RSD1030 were recently reported by Barrett et al [96] in a similar study, and their results were very similar to those reported here. Thus, the use of this model as a means of evaluating the antiarrhythmic properties of test compounds in the context of ischaemia-induced arrhythmias was appropriate.

The results of these experiments yielded antiarrhythmic dose-response curves for protection against ischaemia-induced arrhythmias for each test compound. By fitting these curves using non-linear regression three indices of antiarrhythmic activity were measured: potency, slope, and goodness of fit to a predefined equation describing the dose-response relationship. Since this study sought to determine the characteristics of drug action that confer superior antiarrhythmic protection in the therapeutic setting, the qualities of a useful therapeutic agent had to be defined and quantified. These three variables were selected (potency, slope, and goodness of fit) because they describe properties of drug action that may be of primary importance in the therapeutic setting. The rationale for this will now be discussed.

It is not difficult to define what characteristics make an "ideal" antiarrhythmic drug in the therapeutic setting. Obviously, an agent capable of maximally suppressing arrhythmias at doses producing no undesirable effects would be useful. By this definition, potency is less important than therapeutic ratio (i.e. the potency ratio between beneficial versus adverse effects) and for this reason, potency was not the only variable considered in this study.

In terms of adverse drug effects, current antiarrhythmic drugs present a paradox: it is well-established that many such agents are also proarrhythmic. Furthermore, the mechanisms of pro- and antiarrhythmic action appear to depend on the same electrophysiologic effects (i.e.

slowing conduction and/or prolonging repolarisation). The difficulty thus relates to how one may distinguish between pro- and antiarrhythmic effects. The dose-response curve for arrhythmia score reflects an *indirect* action of drug, based on direct effects on conduction and repolarisation. Furthermore, the endpoints used to measure pro- and antiarrhythmic effects are the same (e.g. a measure of arrhythmic history such as an arrhythmia score). One can therefore appreciate that the dose-response curve for arrhythmia score may reflect *both* pro- and antiarrhythmic actions.

The nature of the arrhythmia score dose-response curve is such that combined pro- and antiarrhythmic drug effects will be reflected in the data by increased variability about the curve for arrhythmia score. The net result will be poor goodness of fit, since the regression equation describes antiarrhythmic effects only (or, more specifically, describes the direct effects of drug on a single variable). Furthermore, the effect of any proarrhythmic tendencies will be to lower antiarrhythmic endpoints at doses where pro-arrhythmic effects become significant. This will serve to decrease the slope of the dose-response curve.

Support for this idea comes from computer simulations performed in the laboratory which produced theoretical antiarrhythmic dose-response curves based on theoretical summations of anti- and pro-arrhythmic effects. The results of such simulations suggested that sigmoidal curves were produced when the proarrhythmic ED_{50} was much greater than antiarrhythmic ED_{50} (i.e. drugs were more potent for antiarrhythmic effects). Aberrant curves, which showed poor goodness of fit to sigmoidal equations, were observed when potencies for the two effects were similar. Furthermore, the slope of the antiarrhythmic dose-response curve was directly related to the extent of overlap between pro- and antiarrhythmic effects (see [124] for discussion).

Further support for this idea comes from an experiment reported in Barrett et al [124]. Ten pharmacologists were given dose-response curves exhibiting various degrees of slope, efficacy, and variability about the dose-response curves. These subjects were not informed as to

which drugs were represented by the dose-response curves and were asked to rank the curves according to their perception of how well the data fit a classic sigmoidal dose-response curve. The results of this subjective analysis were very similar to regression analysis rankings, and for RSD1030, RSD995, RSD988, and RSD944 rankings were identical between the subjective ranking in that study, and the ranking due to regression analysis in the present study.

For the above reasons, slope and goodness of fit were interpreted in this study as indices of antiarrhythmic protection since, in the context of the above, they reflect variability about the dose-response curve and thus specificity for antiarrhythmic effects. With these assumptions, steep slopes and strong regression coefficients reflect drugs which are more specific for antiarrhythmic effects. Conversely, low slopes and poor goodness of fit reflect a decreased specificity for antiarrhythmic effects, possibly due to proarrhythmic tendencies within these dose ranges. These variables have previously been used for such studies [124].

4.3 Electrical Stimulation Experiments

The use of the rat model of electrically-induced arrhythmias in this study achieved two goals. First, it provided a means of evaluating the antiarrhythmic profile of test compounds in the context of non-ischaemic arrhythmias. This was an important control for testing the hypothesis that ischaemia-selectivity is a mechanism for antiarrhythmic protection (and therefore should have no bearing on protection against arrhythmias occurring in the absence of ischaemia). Secondly, it allowed for evaluation of the electrophysiologic actions of test compounds in non-ischaemic cardiac tissue based on observed changes in ECG intervals and stimulation variables.

The use of ventricular fibrillation threshold, VFt, as an index of antiarrhythmic protection is based on the mechanism of initiation of VF whereby premature activation of the ventricles occurs during the vulnerable period of late systole when repolarisation is spatially and temporally heterogenous [175]. Thus, drugs which increase VFt presumably do so by reducing

excitibility of the membrane (i.e. blockade of sodium current) or by prolonging repolarisation in a manner that decreases heterogeneity of refractoriness throughout the ventricular myocardium. The complexity of this mechanism relates to the fact that slowing of conduction can have effects on both the size and the shape of the vulnerable period. This translates into decreasing the current threshold for induction of VF, and prolonging that portion of the cardiac cycle during which VF may be stimulated, respectively. Thus, drugs may be antiarrhythmic and/or proarrhythic based on their differential effects on these parameters. Computer modelling studies by Fishler and Thakor [176] suggest ischaemia further affects the size and shape of the vulnerable period in a time-dependent manner.

Since inducing ventricular fibrillation is often irreversible there is a need for electrical defibrillation with DC voltage if the experiment is to continue. In order to minimise the effects of repetitive defibrillation on experimental preparations the use of ventricular fibrillo-flutter as an appropriate measure of vulnerability can be substituted for true VF, especially in rats [177]. Fibrillo-flutter appears on the electrocardiogram as waveforms with characteristics of both tachycardia and fibrillation, and is accompanied by a precipitous drop in blood pressure that may not fall to zero as in true VF. The use of serial shocks to induce ventricular fibrillo-flutter has been described previously for the conscious feline [178], anaesthetised rat [179], and isolated perfused rat heart [180]. In those experiments, ventricular fibrillo-flutter thresholds were found to correlate linearly with ventricular fibrillation thresholds.

In this study a serial shock method was used, based on that described previously [181;182] to induce ventricular fibrillo-flutter in pentobarbitone-anaesthetized rats. This model has been used extensively for antiarrhythmic drug research [173;177] and control results in this study agreed with those of previous investigators [181;182]. Compounds in this study all had effects on VFt. This was attributed to either class I or class III effects, or a combination of both

(see below). Potency indices for effects on VFt for four of the compounds studied here were very similar to those reported elsewhere [124].

To explore the electrophysiologic effects of test compounds in non-ischaemic myocardium the ECG and various other electrical stimulation variables were measured. The ECG is a summation of cellular events within the entire heart and as such does not allow for the distinction of individual components of the cellular action potential. However certain measures are useful for estimating effects on conduction and repolarisation.

The PR interval and QRS duration are indicators of conduction velocity in the AV node and ventricles, respectively. Ranger et al [183] showed in humans that QRS was a reliable measure of the effects of sodium-channel blocking drugs on V_{max} of the action potential and thus reflected blockade of sodium current. As considered by Barrett [96], measurements of drug effects on these variables assume that the path of conduction does not change after drug treatment. The protocol used here controlled for this by using cumulative dosing regimens: if effects were merely due to a change in conduction pathway after administration of drug, one would not expect to see dose-dependent changes in conduction, although effects relative to pre-drug might be exaggerated. In this study PR was more sensitive to the action of drugs than QRS, which in many cases did not change significantly enough to be accurately fit to the dose-response equation. This is perhaps due to the fact that, in the rat, the QRS interval is quite small and often fused to the T-wave such that Q and S can be more difficult to identify than P and R [163]. This occurs due to the rapid repolarisation phase that follows depolarisation in the rat, resulting in some ventricular cells repolarisating during the QRS wave of the ECG [184].

The P-wave represents speed of conduction between the atria and ventricles. This depends on conduction through atrial and ventricular myocytes, a function of sodium channel availability, but more importantly, conduction through the AV node. The latter type of conduction can be slowed by block of inward calcium currents, which are responsible for

activation in AV nodal cells. Thus both sodium and calcium channel blocking drugs may increase the PR interval. In this study, changes in PR interval were attributed to inhibition of sodium current because previous studies with related compounds have shown them to be largely mixed sodium and potassium channel blockers [116;123].

Another means of assessing drug effects on excitability was used in this study: measurement of iT, the threshold current required for capture in pacing regimens. This variable relates to the ability to stimulate ventricular tissue and is therefore dependent largely on sodium channel availability [185]. The D25% values for iT in this study were similar to those for PR (see Table 7). Thus, decreases in PR interval and iT were interpreted in this study as sodium current inhibition.

The use of the QT interval to measure effects on repolarisation is controversial [186]. It is particularly difficult in rat hearts, where action potential duration is short and where the T-wave is more closely associated with the QRS complex than in other species. For this reason effects on ERP and MFF were also measured to estimate effects of drug on repolarisation. ERP and MFF measure the refractory period by determining the minimum S1-S2 interval that can provoke a premature response, and the maximum frequency at which the heart can be paced, respectively. In normal myocardium refractory period is closely associated with repolarisation, unlike in ischaemic tissue where post-repolarisation refractoriness occurs. Prolongation of refractoriness can occur by blockade of repolarising outward potassium currents or by drug-induced delay of sodium channel recovery from inactivation [185]. In this study, potency indices for QT1, QT2, ERP, and MFF were very similar for any particular drug and showed significant correlations with one another (see Table 7). In antiarrhythmic drug research prolongation of QT interval is usually interpreted as corresponding to prolongation of repolarisation [187].

In this study QT Interval was measured in two ways. The difference between QT1 and QT2 relates to the nature of the prolongation of the T-wave (see Figure 2). In arterially perfused

canine wedges the T wave represents dispersion of repolarisation times between epicardial and M-cells [188]. The peak of the T-wave was related to the earliest point of repolarisation, which was observed in epicardial action potentials. Repolarisation in M-cells, which took longest time, corresponded with the return of the T-wave to the isoelectric line. Thus comparing drug effects on QT1 versus QT2 may provide insight into the specific nature of drug effects on dispersion of repolarisation. However, in the present study no significant differences were observed between potency for QT1 and QT2 prolongation for any compounds. Studies have shown significant regional differences in I_{to} distribution in rat ventricular myocytes [189] however these differences may be far less significant in whole animals where the effects of electrotonic coupling are to reduce heterogeneities throughout the myocardium [188].

4.4 Isolated Heart Experiments

4.4.1 Evidence for Ischaemia

The isolated occluded rat heart model has been used by many investigators to examine the effects of antiarrhythmic drugs on arrhythmias induced by regional ischaemia [190-193]. For a review of this method see Curtis [194].

To ensure that sufficient levels of ischaemia were established, the occluded zone was measured at the conclusion of each experiment (see Methods). It was confirmed upon staining that the recording region fell well within the boundaries of the ischaemic zone, and that recordings from normal tissue were not contaminated by ischaemic tissue (not likely since the normal region recording area was on the opposite face of the heart relative to the ischaemic zone). This highlights one of the advantages of optical recording over conventional monophasic action potential methods: since electrical activity is only detected at sites illuminated by excitation light there is no possibility that remote activity will be detected in local recording areas.

Further evidence of ischaemia came from the observed changes in optical action potentials following occlusion. In the occluded zone of control hearts ischaemia caused a reduction in amplitude and rise rate of the action potential and a decrease in APD consistent with reports of effects of ischaemia on monophasic [62] and intracellular [195] action potentials measured using conventional methods. Sham-treated hearts showed no changes in action potential characteristics between perfused and sham-occluded regions, whereas significant differences were seen in occluded hearts. This provides strong evidence that sufficient degrees of ischaemia were produced in the region from which occluded-zone action potentials were recorded, and not in regions where perfusion was assumed to remain normal.

An interesting observation was that APD_{25} was prolonged in ischaemia, whereas APD at 50, 75, and 90% repolarisation was shortened. Barrett et al [96] reported no such observation in studies with rabbits, however this may be due to the different morphologies and ionic repolarisation currents in rabbit versus rat ventricular action potentials. Inoue et al [195] observed similar effects of ischaemia on intracellularly recorded action potentials in rats *in vitro*. Figure 1B in that publication shows ischaemic action potentials with a distinct widening of the earliest phase of repolarisation compared to action potentials recorded in normally perfused myocardium, while the mid- and later phases of repolarisation show obvious shortening. No explanation has been put forward for this phenomenon.

4.4.2 Selection of time point at which to analyse ischaemic action potentials

The choice of three minutes as the time during ischaemia when measurements were made was based on several observations. Firstly, the signal-to-noise ratio deteriorated with time during ischaemia. Secondly, experimental evidence shows that the effects of ischaemia are significant by three minutes post-occlusion [164], including elevation in potassium concentration [74] and decrease in extracellular and intracellular pH [91]. Thirdly, the occurrence of arrhythmias

interfered with measurements to the least extent at this time point. In most hearts, VT and VF did not occur prior to three minutes post-occlusion, and in the few hearts where they did occur these arrhythmias were of short duration (less than 15 seconds) and did not occur within one minute of the time at which action potential measurements were taken. This minimalised the effects of restitution and memory, two properties of cardiac tissue which affect action potential duration upon a change in rate. By four to five minutes many hearts had experienced more severe arrhythmias. Alternans almost exclusively occurred during the first minute after occlusion, and did not occur during the time period in which samples were analysed. Obviously, action potential measurements could not be made during arrhythmias. Thus, three minutes post-occlusion was chosen as the time point at which to measure effects on action potentials during ischaemia. The limitations of this will be discussed in section 6 below.

4.4.3 Measuring Excitability Using Optical Action Potentials

Slowing of conduction in ventricular myocardium may be affected by depolarising inward currents (i.e. sodium), the spatial pattern and degree of gap junctional coupling, and the geometry of cell and fibre orientation [196]. In this experiment the optical action potentials recorded were multicellular. As with conventional multicellular recording techniques the rise rate of the action potential may be considered to represent not only sodium current availability but also the degree of intercellular resistance (i.e. gap junction coupling). Delays in conduction time will "blur" the upstroke of a multicellular action potential, causing the apparent rise rate to decrease. The advantage of optical recording techniques is that conduction velocity may be precisely determined because the size of the recording area is precisely known (it is the dimensions of the photodiode divided by any magnification). Thus if conduction time is measured (time from stimulus to the start of the action potential), the speed of conduction can be

accurately determined and effects of changes in intercellular coupling can be related to observed changes in maximum upstroke velocity.

It was not necessary to estimate speed of conduction in the present study to account for possible changes in intercellular coupling. The test compounds were from a family of related compounds that have been previously shown to block sodium currents in rat ventricular myocytes [116]. While the effects of these drugs on gap junction resistance have not been tested, their potency for blocking the inward sodium current is proportional to the degree of slowing of conduction observed, for series of related compounds with a range of such potencies [personal communication, Nortran Pharmaceuticals]. Thus, for effects in normal tissue, comparison of drug-related changes in V_{max} relative to pre-drug values are assumed to reflect only changes in sodium channel availability. This also assumes that gap junctional resistance does not change over the course of the experiment in the normal region. For this reason drug effects were also compared to non-drug treated hearts so that any time-dependent changes in V_{max} would also be controlled for.

The effects of ischaemia on gap junction resistance have been well-documented (see Jongsma and Wilders [65] for a recent review). Ischaemia increases intercellular resistance by affecting gap junction density [197] and distribution [198]. For this reason, drug effects on V_{max} were related to pre-drug values, and control values in vehicle-treated hearts, for ischaemic tissue. In this way effects of cell-to-cell coupling on maximum upstroke velocity were controlled for such that changes in V_{max} in drug-treated hearts could be reasonably assumed to relate to drug-induced changes in cell excitability (i.e. sodium channel availability) and not changes in intercellular coupling.

4.4.4 Arrhythmias in the Isolated Heart Model

In the isolated heart model, arrhythmias were frequently observed after occlusion of the left coronary artery. These ranged in severity from premature beats, to VT and VF, both reversible and non-reversible (ie. greater than three minutes continuous duration). Alternans was also frequently observed, consistent with reports of other investigators [96]. However, arrhythmias were not recorded during experiments performed here.

To compare the incidence and severity of arrhythmias in isolated heart experiments to those of coronary occlusion experiments, and to quantify them using the same arrhythmia score, it would have been necessary to record data continuously for fifteen minutes. Due to the photobleaching effect inherent to the optical action potential recording technique, this would have resulted in significant signal deterioration, as shown in pilot experiments (see Results). Limitations of data acquisition systems were also a consideration. The system used was not programmed to acquire data from more than three channels simultaneously which precluded acquisition of the ECG for quantification of arrhythmias. Also, recording four channels at the high sampling rates used (sampling rate could not be varied between channels) would have resulted in very large data files, taxing the memory capacity of the data acquisition computer and considerably slowing automated analysis procedures. The costs of re-programming the system to include more channels and upgrading the computer to one with faster processing speeds and greater memory capacity were considerable.

The incidence and severity of arrhythmias in isolated heart models has been well-documented [194] and since it has been convincingly shown that these do not preclude this model from being an effective technique for detecting antiarrhythmic activity, it was not considered necessary to quantify arrhythmias. This does, however, represent a possible limitation of this study and this will be addressed below (see section 4.6).

4.4.5 Quantifying Ischaemia Selectivity

Three different methods were used to determine selectivity for effects on V_{max} and APD_{50} in ischaemic versus normal tissue. The Response Ratio (RR) method based ischaemia-selectivity on the effects of a single concentration, in this case the highest. The limitations of this technique are that the proportion of effects in normal and ischaemic tissue were not the same at the two concentrations tested. Thus the ranking of compounds based on ISR would be different depending on whether the low or high concentration was used in the calculations and it is not possible to determine which more accurately represents the true degree of ischaemia-selectivity. The highest concentration was chosen because, for the majority of drugs, effects were significant in both normal and ischaemic tissue at this concentration.

The Parallel Shift and Potency Shift methods were an attempt to measure actual potency shift using extrapolations of two-point curves to a given response. Extrapolations of x from regression equations is more accurate than extrapolations of y because the latter is a dependent variable [129]. Thus, in pharmacological research, estimations of true potency shift are considered more appropriate than estimations based on response ratios when determining the effects of an intervention (i.e. ischaemia, or an antagonist drug). Both methods assume equal slopes for the two curves (normal and ischaemic). In the Parallel Shift method, the value of the slope was not considered. This result may not be reflective of the data if the slopes are significantly different for normal and ischaemic responses.

The Potency Shift method was introduced to control for these differences by estimating the actual value of slope using data which showed a significant increase from control (and thus could be reasonably assumed to fall on the linear portion of the dose-response curve). The limitations of this were that slope was assumed to be the same for all drugs. Since the test compounds possessed varying degrees of potency with respect to effects on conduction and

repolarisation, it would be reasonable to suggest that the slope for effects in normal or ischaemic tissue might also have varied between compounds.

Each method for determining ISR had its own advantages and limitations. The primary limitations were due to the nature of the data itself. For example, RSD1031 produced no effects on V_{max} in either normal or ischaemic tissue compared to controls, thus calculations of ISI-V using either method were not indicative of actual ischaemia-selectivity for this compound. It was not possible to use higher concentrations because effects of this drug on repolarisation in ischaemic tissue were significant and in pilot experiments it was difficult to maintain capture at higher concentrations while maintaining the rate of 7.5 Hz. This situation would be expected to occur with any compound where the potency ratio for effects on conduction and repolarisation was large. Ideally, dose-response curves would be constructed for each compound using a range of concentrations but since the supply of drug was limited it was not possible to do so.

The three methods were evaluated for their usefulness in assessing the effects of ischaemia on drug action. It was noted that the significance of a particular value differed between methods. For example, in the Response Ratio method the value of the number indicated whether the drug was more selective for normal tissue (ISI < 1) or ischaemic tissue (ISI > 1). In the Parallel Shift and Potency Shift methods, a negative value indicated that effects in normal tissue were greater than effects in ischaemic tissue and a positive value indicated the opposite. Furthermore, the spread of ISI values was much greater for the Potency Shift method (from –8.9 to 25.5) than for the other methods (-2.19 to 4.13 and –0.57 to 7.27 for Parallel Shift and Response Ratio methods, respectively). Therefore it was appropriate to evaluate the methods based on rank order of ischaemia-selectivity, rather than absolute values. The following sections discuss ISI values from different methods (Tables 9 – 11) in relation to the raw data shown in Figures 13 (for ΔV_{max}) and 14 (for ΔAPD_{50}). ISI-V for RSD1031 was not considered since, as noted above, effects in both normal and ischaemic tissue were no greater than controls.

4.4.5.1 Estimation of Ischaemia-Selectivity for Effects on V_{max}

Using the Response Ratio method, an ISI-V value less than 1.0 demonstrated that drug effects on V_{max} were greater in normal tissue than in ischaemic tissue. This was the case for four out of five test compounds for which ISI-V could be determined. Figure 13 shows that these drugs (RSD944, RSD988, RSD995, and RSD1020) did in fact show greater effects in normal tissue than in ischaemic tissue, relative to control values. Using the Parallel Shift and Potency Shift methods, a negative ISI-V indicated greater selectivity for normal tissue. The same four compounds above had negative values in both methods. Thus, all three methods successfully detected compounds that were more potent in normal tissue, and identified the one ischaemia-selective drug, RSD1030.

The differences in rankings for ISI-V (see section 3.4.4) stem from the rank order of the four compounds that were selective for normal tissue. Which one best reflected the data? Estimations of ischaemia-selectivity were made by examining the data in Figure 13. The slopes of the dose-response curves for normal and ischaemic effects were assumed to be equal for a single compound. The potency shift was thus determined by the steepness of the slope and the separation between normal and ischaemic responses.

In Figure 13, RSD995 appeared to have the steepest slope, and the difference between normal and ischaemic responses was large. Therefore, selectivity for normal tissue for this compound appeared to be the highest. RSD1020 also appeared to have a fairly steep slope, but the differences between ischaemic and normal responses were less. The slope for RSD988 was a bit shallower than that of RSD1020 but the differences between normal and ischaemic responses were greater, suggesting that selectivity for normal tissue for RSD988 was greater than that of RSD1020. Unfortunately, the data for RSD944 does not suggest the slope, since values were similar for both concentrations. This meant that either slope was very shallow, or the two

concentrations tested represented the left plateau of the sigmoidal dose-response curve (this is discussed further in section 6 below).

To clarify this, the slopes for responses in normal tissue from electrical experiments were considered. While data for slope were not reported for these experiments, examination of the raw data showed that slope for RSD944 for effects on iT was significantly steeper than that of RSD1020 and closer to that of RSD995. Furthermore, as seen in Figure 13, effects on normal tissue are not much greater than effects in ischaemic tissue, similar to RSD1020. From this it was estimated that selectivity for normal tissue for RSD944 was greater than that for RSD1020, less than that for RSD995, and similar to, but less than, that of RSD988. Thus, from Figure 13 it was estimated that order of selectivity for normal versus ischaemic tissue, from least to greatest, was RSD1020 < RSD944 < RSD988 < RSD995. This was closest to the rankings of the Response Ratio method, which yielded RSD944 < RSD1020 < RSD995 < RSD988 (note that the rank orders shown in section 4.5 represent selectivity for ischaemic tissue whereas above the order is reversed to highlight selectivity for normal tissue).

Interestingly, the ranking of RSD1030, RSD995, RSD988, and RSD944 based on ISI-V as determined by the Response Ratio method was the same as in a similar study using simulated ischaemia [124]. On the other hand, in the latter study all these compounds were found to be slightly ischaemia-selective, whereas in this study they were found to be normal-selective. This may be due to the different techniques used to quantify ischaemia-selectivity and the fact that PR was used, whereas in this study effects on V_{max} were used.

4.4.5.2 Estimation of Ischaemia-Selectivity for Effects on APD_{50}

The ISI-R values calculated using the three methods above showed that all six compounds were more potent for effects on APD_{50} in ischaemic than in normal tissue. All methods ranked RSD1020 as the most ischaemia-selective for effects on APD_{50}. RSD1030 also

ranked high for ischaemia-selectivity, but the rankings of the other four compounds were considerably different.

In Figure 14, it was easier to visualise the relative potencies for ischaemic tissue because effects in control hearts were small in comparison. It was apparent that for 1.0 μM RSD1020, effects in normal tissue were a very small fraction of those in ischaemic tissue. RSD1031 at its highest concentration (4.0 μM) was more ischaemia-selective than RSD1030 (at 3.0 μM), based on the significant effect of the latter drug in normal tissue. The remaining drugs were ranked as RSD995 > RSD988 > RSD944 in decreasing order of ischaemia-selectivity. However, these are obviously the same calculations as those used to determine ISI-R with the Response Ratio method. Was there more information to consider?

Using the same procedure as that above for Figure 13, slopes were estimated based on responses in both regions to both concentrations. By considering the difference between normal and ischaemic responses it became apparent that RSD944 had the steepest slope and the least separation between normal and ischaemic responses. RSD988 had a shallower slope, similar to that of RSD1030 but the latter had a much greater degree of separation between normal and ischaemic effects. The slopes of RSD1020 and RSD1031 were similar, as was the separation between normal and ischaemic effects. RSD995 did not have a large separation between effects, however the slope was rather shallow. This explained the results of the Parallel Shift method which ranked RSD995 in second place for ischaemia-selectivity. The Potency Shift method ranked RSD1030 as equivalent to RSD944, but this did not seem to be supported by Figure 14.

These discrepancies between methods may be explained by the fact that, for effects on APD_{50}, there were no cases in which responses in normal and ischaemic regions were signficantly greater than control for both doses. In Figure 13, there were three drugs where this was the case. Thus the Potency Shift and Parallel Shift methods would be expected to be less dependable, since they both assume equal slopes and data that falls on the linear portion of the

dose-response curve and this was not the case for any drug with respect to effects on APD_{50}. Once again, the limitations can be attributed to the data, and not to the methods. However, for this group of data it appeared that the Response Ratio method was the best at determining the rank order of ISI-R for the six test compounds.

4.5 Discussion of the Results

4.5.1 Effects on Blood Pressure

Drugs which slow conduction in non-ischaemic cardiac tissue may also have effects on neuronal conduction in smooth muscle by virtue of their sodium channel blocking properties, and this could result in hypotensive effects. This suggests that drugs which are selective for ischaemia with regards to effects on conduction will produce limited effects on blood pressure at doses that suppress ischaemic arrhythmias. To explore this possibility we measured potency indices for hypotensive effects (decrease in MAP).

The only significant correlation found for D25% for MAP was a positive relationship with potency for QT1 Interval prolongation. It is unclear how prolongation of repolarisation in vascular neuronal or endothelial cells could cause hypotension, but there is reason to doubt the significance of this correlation. D25% for MAP did not correlate with any other variable for effects on repolarisation, even though all four repolarisation indices were strongly correlated with one another. Furthermore, examination of the data showed that QT1 Interval could not be estimated for one compound, RSD995. This happens to be the only compound that showed no detectable effects on MAP. This skewed the results of correlation analysis in favour of a positive correlation, as evidenced by the fact that data for MFF was also missing an estimate, but for a different compound, and there was no positive correlation. Thus it would seem that the positive relationship between QT1 and MAP was correlative, and not causative, in nature.

The mechanism for reduction in mean arterial pressure is unclear from the data. For RSD944 and RSD988 effects on MAP could be related to effects on conduction slowing and occurred at doses similar to those for antiarrhythmic protection. On the other hand, RSD995 showed no effect on MAP but was still potent for conduction slowing. This finding is in conflict with results reported by Barrett et al [124] who showed that RSD995 was more potent at reducing blood pressure than RSD988, RSD944, or RSD1030. For the latter three compounds, however, their results agree with the D25% values in this study.

For RSD1020 effects on MAP were significant and occurred at doses similar to those for antiarrhythmic protection, but at much lower doses than potency for conduction slowing. For RSD1030 and RSD1031, effects on MAP were similar to doses for conduction slowing, but these were very high doses. Thus, for these compounds effects on MAP appeared to be due to a variety of mechanisms which may explain why an inverse correlation between ischaemia-selectivity and potency for MAP effects was not found. While Yong [116] was able to show a correlation between therapeutic ratio (ratio of potencies for MAP, HR, and LD_{50} with ED_{50} for antiarrhythmic protection) and selectivity for antiarrhythmic effects (slope and goodness of fit variables), he was not able to ascribe these effects to any particular ionic mechanism.

4.5.2 Antiarrhythmic Protection Against Ischaemia-Induced Arrhythmias

Based on the antiarrhythmic dose-response curves for protection against ischaemia-induced arrhythmias, the test compounds could be grouped into roughly three categories: those with steep slopes and strong goodness of fit values (RSD1020 and RSD1031), those with more shallow slopes and smaller goodness of fit values (RSD1030, RSD995, and RSD988), and those that did not fit the equation (RSD944). Data in Table 4 suggest that the dose-response curve for RSD944 reflected a lack of dose-dependent effects on VF, but not VT which was reduced at the

highest dose. The rank order of compounds for the three variables measured – slope, goodness of fit, and potency – reflected this grouping.

Rank order for slope and goodness of fit were almost identical to one another, with the only difference being in the first and second place ranking which included the "best" drugs as determined by the criteria above. However, a significant positive correlation was not found using correlation analysis. Examination of the data suggests that this was due to the steep slope of RSD1020, which had a smaller relative r^2 value. This one point was a strong enough "outlier" that the correlation was not significant. Interestingly, if rank correlation analysis is performed for these two variables, a significant relationship is found ($r_S = 0.943$ versus $r_{S,critical} = 0.886$). This is consistent with the idea that slope and goodness of fit represent the same characteristic (i.e. variability about the dose-response curve).

The rank order for potency against ischaemia-induced arrhythmias was opposite to that for slope and goodness of fit. Correlation analysis showed a significant inverse relationship between ED_{50} and r^2. In other words, drugs with highest potency showed the greatest variability about the dose-response curve, suggesting that such compounds had proarrhythmic effects over the dose range studied. It has been noted above that, in terms of what constitutes a desirable therapeutic agent, potency may come secondary to considerations of therapeutic ratio (i.e. unwanted effects at doses producing desired effects). Therefore, these results suggest that in this study and with these particular compounds, slope and goodness of fit define the ideal therapeutic antiarrhythmic agent better than considerations of potency alone. While there is no reason to suppose that potency could not correlate with slope and goodness of fit, in this study they were opposite, which supports the idea that potency should not be the only criteria for defining "ideal" therapeutic antiarrhythmics.

Where characteristics of potency and variability of the dose-response curve differ, the latter may be considered more important. Interestingly, Yong [116] found no correlation between

potency for protection against ischaemia-induced arrhythmias and pK_a or log Q, indices of physicochemical properties that correlated with more effective antiarrhythmic protection. It is interesting to note that the three "best" compounds in this study (RSD1020, RSD1030, and RSD1031), in terms of effective antiarrhythmic protection, had pK_a values that were near neutral (all around 7.4) whereas the less effective compounds (RSD995, RSD988, and RSD944) had pKa values around 9.4. Thus, drugs which were already largely protonated (considered the active form of the drug) at normal and acidic pH had less effectiveness than drugs which were only largely protonated at acidic pH. This suggests a mechanism for the ischaemia-selectivity observed with these compounds.

While drugs with steep slopes and strong goodness of fit values showed less potency, they did not show less efficacy. Examination of Figure 5 shows that the least potent drugs tended to show responses that reached a maximum arrhythmia score of zero in a majority of rats at the highest doses. On the other hand, the most potent compounds showed less efficacy. These results can only be interpreted as a trend towards less efficacy with greater potency, since regression analysis did not reveal a significant difference in y_{max} between these compounds (except for RSD944, which could not be fit to the regression). However, Barrett et al [124] did find a significant relationship between efficacy and ischaemia-selectivity in a similar study with related compounds.

The above supports the use of slope and r^2 as indices of antiarrhythmic effectiveness in this study, because drugs with steep slopes and high r^2 values showed maximum safety and efficacy, even though they were less potent.

4.5.3 Mechanisms of Antiarrhythmic Protection

4.5.3.1 Protection Against Non-Ischaemic Arrhythmias

A positive correlation was found between D25% values for VFt and MFF. It is reasonable to suggest that prolongation of repolarisation in normal tissue may be a mechanism for protection against non-ischaemic arrhythmias. While Kwan et al [199] showed that VFt was not affected by class III effects alone these drugs may not act in the same way as traditional class III drugs. In this study, potency for prolongation of repolarisation was close to or less than potency for VFt for all compounds.

For RSD995, RSD988, and RSD944, their potency for effects on repolarisation and excitability were roughly equal therefore effects on conduction in normal tissue, or mixed effects on both conduction and repolarisation, cannot be ruled out as a mechanism of antiarrhythmic protection for these compounds. These results are similar to those previously reported [124].

On the other hand, potency for PR Interval was much greater than potency for VFt for RSD1020, RSD1030, and RSD1031 suggesting that reduction in excitability was not a mechanism for reducing VFt for these compounds. These compounds did have effects on repolarisation indices that were more potent than effects on PR and iT, but repolarisation effects occurred at lower doses than effects on VFt. Barrett et al reported the same results for RSD1030 [124].

In summary, the antiarrhythmic effects of drugs in this series with respect to non-ischaemic arrhythmias appeared to relate to a reduction in maximum following frequency. This may have been mediated by inhibition of sodium current however, effects on VFt occurred at doses that corresponded more closely to those for prolongation of QT interval, suggesting the mechanism may in fact be prolongation of action potential duration. There is also the possibility that drugs with strong potencies for VFt were more protective due to mixed channel-blocking effects.

4.5.3.2 Protection Against Ischaemic Arrhythmias

A positive correlation was found between ED_{50} (protection against ischaemic-arrhythmias) and D25% for VFt. While the range of values for ED_{50} were smaller than those for D25%, this could be explained by the fact that D25% values may represent a different point on the dose-response curve. D25% for VFt was calculated with the assumption that all drugs tested had equal efficacy. If this was not the case, it would be expected that D25% values would vary in their position on the dose-response curve relative to ED_{50} values. This suggests that the correlation may represent a real phenomenon and not just a reflection of potency order.

Both ED_{50} and VFt correlated with potency for prolonging refractoriness (as measured by MFF). Does this suggest that protection against ischaemic and non-ischaemic arrhythmias share the same mechanism? It is doubtful that effects in normal tissue alone would be protective against ischaemic arrhythmias. Barrett et al [125] showed that the antiarrhythmic effects of a related compound, RSD1019, could not be attributed to its effects on repolarisation in normal tissue. Examination of ISI-R data reveals that all six compounds were preferentially selective for effects in ischaemic tissue, albeit to differing extents. Thus, drugs which had effects on repolarisation in normal tissue also had effects in ischaemic tissue that were equivalent or greater. This would explain the correlation between ED_{50} and MFF, and provide a mechanism by which these compounds protect against ischaemic arrhythmias – by prolongation of repolarisation in ischaemic tissue.

4.5.4 Mechanism of Selectivity for Antiarrhythmic versus Proarrhythmic Effects.

It has been discussed at length above that slope and goodness of fit to the antiarrhythmic dose-response curve likely reflect specificity for antiarrhythmic versus proarrhythmic effects. The "ideal" antiarrhythmic drug has been defined as having optimal slope and r^2 values, irrespective of potency. The central hypothesis of this thesis is that drugs which are more potent

in ischaemic tissue will be better antiarrhythmic drugs than those that have significant effects in normal tissue. Thus, slope and goodness of fit should correlate with ischaemia-selectivity, and be inversely correlated to effects in normal tissue.

All drugs in this study showed increased potency for effects on repolarisation in ischaemic versus normal tissue. How does this explain the poor slope and r^2 values for some of these compounds? The answer lies in the ischaemia-selectivity values for effects on V_{max} and the relative potencies of these compounds for effects on excitability versus repolarisation.

ISI-V values show that four of the five compounds were more selective for normal tissue in terms of effects on excitability. Three of these compounds - RSD995, RSD988, and RSD944 - were equally potent for effects on conduction and repolarisation in electrical stimulation experiments, and were more potent than the other compounds. Thus, in the context of ischaemia, these drugs have significant effects on both excitability and repolarisation in normal tissue, with less effect on excitability in ischaemic tissue, and only marginally greater effects on repolarisation. The net effect of this is expected to be increased proarrhythmic tendencies. This was reflected in the poor slopes and goodness of fit values for these drugs. These results are consistent with those of Barrett et al [124] who reported that these compounds had poor slope and r^2 for arrhythmia score dose-response curves, and this was related to lack sufficient selectivity for conditions of simulated ischaemia, and not lack of potency in normal tissue.

RSD1020 was also more selective for normal tissue in terms of effects on V_{max}. However, this compound was associated with a steep slope and high r^2 value. This apparent contradiction can be explained by the fact that RSD1020 was significantly more potent for effects on repolarisation than effects on excitability in electrical stimulation experiments. Furthermore, it was the most ischaemia-selective compound for effects on repolarisation. Thus at doses which caused significant prolongation of repolarisation in ischaemic tissue and relatively little effect in normal tissue, effects on conduction were negligible.

RSD1030 was the only drug shown to be ischaemia-selective with respect to effects on V_{max}. It was also highly ischaemia-selective for effects on repolarisation. Since electrical stimulation experiments showed that potency for PR effects was significantly less than potency for repolarisation effects, it can be suggested that RSD1030 protects against ischaemic arrhythmias by virtue of selective prolongation of repolarisation in ischaemic tissue at doses that have little, if any, effect on conduction. However, even if effects on conduction were significant, this drug is highly ischaemia-selective therefore this would not be expected to produce any proarrhythmic effects. RSD1030 showed steep slope and strong goodness of fit in coronary occlusion experiments, consistent with this possibility.

ISI-V for RSD1031 could not be estimated because this drug had no significant effects on V_{max} at either concentration tested, in either normal or ischaemic tissue. However, this compound had significant effects on repolarisation that were very selective for ischaemic tissue at the same concentrations. Therefore under conditions of ischaemia this drug will prolong repolarisation in ischaemic tissue at doses that have no effect on conduction. This agrees with the steep slope and high r^2 value for this drug.

ISI-V was inversely correlated with effects on both PR Interval and repolarisation (ERP, QT2, and QT2). This correlation reveals that drugs which were more selective for normal tissue (i.e. smaller ISI-V values) were more potent at reducing excitability and prolonging repolarisation in normal tissue, whereas the ischaemia-selective drug (RSD1030) was least potent. ISI-V also correlated inversely with VFt. Drugs which were more "normal-selective" were more potent at protecting against non-ischaemic arrhythmias than ischaemia-selective drugs.

On the other hand, ISI-R correlated strongly with both slope and goodness of fit such that ischaemia-selective drugs were more specific for antiarrhythmic versus proarrhythmic effects. All drugs showed increased potency for effects on repolarisation in ischaemic versus normal

tissue, although the degree of selectivity varied widely amongst the six compounds. This represents a unique finding for antiarrhythmic action since most drugs that prolong repolarisation are reverse use-dependent [200] and therefore expected to be less effective in ischaemic tissue. West et al [201] showed that E-4031 and dofetilide had decreased potency for blockade of I_{Kr} under conditions of hyperkalemia and acidosis. Barrett et al [125] showed that tedisamil had decreased potency for prolongation of monophasic APD_{90} in ischaemic versus normal rabbit ventricular myocardium. Thus the RSD compounds may represent a unique group of drugs with Class III actions that are more potent under conditions of ischaemia.

4.5.5 Ionic Mechanisms of Antiarrhythmic Action

This study did not examine the effects of drug on ionic currents directly nor were binding studies performed. It was assumed that effects on PR and iT reflected block of sodium channels, and that effects on ERP, QT1, QT2, and MFF reflected block of potassium channels (specifically I_{to} since it is the primary repolarising current in rat). There is considerable evidence to support these suggestions.

Previous studies have shown that related drugs block I_{Na} and I_{to} [50;116]. Effects on V_{max} of the action potential may be more reasonably concluded as relating to effects on sodium channel availability (see section 4.4.3) and these appeared to agree with effects on PR and iT, though direct comparisons of potency could not be made (see section 4.6). Evidence for block of potassium currents comes from effects on cardiac action potential duration. APD at 25, 50, 75, and 90% repolarisation was prolonged by all drugs in this study. While APD can be prolonged by block of inactivated-state sodium channels, the three most potent compounds for prolonging APD in ischaemic tissue had virtually no effects on V_{max} at the same concentrations. Furthermore, potency for prolongation of repolarisation indices in normal tissue was

significantly greater than that for effects on PR and iT for three of the six compounds, suggesting that effects on repolarisation were not due to effects on sodium channels.

Barrett et al [124] showed that all the RSD compounds they studied were more potent for effects on ERP than PR. ERP effects were attributed to sodium channel block, but because all compounds except 1030 had VFt potencies that were similar to those for ERP, and not PR or QRS or iT, it is possible that effects on ERP reflected both sodium and potassium channel block as all drugs showed significant effects on QT interval at the same dose. Recently, Barrett et al [125] reported that RSD1019 is an effective antiarrhythmic because of selective effects on monophasic APD in ischaemia that were not associated with effects on V_{max}.

The results of this study suggest that prolongation of repolarisation is a mechanism for antiarrhythmic protection and that conduction slowing, particularly in normal tissue, is a mechanism for proarrhythmia. Kirchhof et al [202] showed that both procainamide and propafenone were antiarrhythmic by virtue of prolongation of post-repolarisation refractoriness in isolated rabbit hearts. However, propafenone was also proarrhythmic due to use-dependent conduction slowing effects, even though it produced greater post-repolarisation refractoriness than procainamide. This shows that antiarrhythmic potency can be offset by proarrhythmic tendencies.

A mechanism for proarrhythmia due to sodium channel block in ischaemic tissue has been suggested. Differential effects of sodium-channel blocking drugs on transverse and longitudinal propagation may be important in anisotropic tissues (e.g. ischaemia) and promote reentry by facilitating conduction block or increasing dispersion of repolarisation [203]. Another mechanism has been suggested by Starmer et al [109] based on theoretical models of conduction slowing in ischaemic tissue. They found that the antiarrhythmic effects of conduction slowing could be attributed to a delay in appearance of the "vulnerable window" for development of reentrant circuits following the onset of ischaemia. However, the "price" for this antiarrhythmic

efficacy was increased proarrhythmic vulnerability due to a concommitant widening of the duration of the vulnerable window. Yin et al [16] concluded that the proarrhythmic effects of lidocaine were due to rate-dependent increased conduction delay and block while antiarrhythmic effects were due to rate-*in*dependent block of the reentrant impulse by prolonged refractoriness.

The antiarrhythmic mechanism of prolonging of action potential duration in ischaemic tissue is due to the fact that recovery of excitability occurs at negative membrane potentials and therefore prolonging APD will be expected to prolong post-repolarisation refractoriness. A longer refractory period requires that, in order for a reentrant circuit to develop, conduction must be slowed to an even greater extent, since the wavelength of a reentrant circuit is defined as the product of conduction velocity and refractory period. Thus prolonging APD in ischaemic tissue may increase the requirements of conduction slowing such that tissue becomes inexcitable before reentry can occur.

Prolongation of action potential duration may also be proarrhythmic if it enhances dispersion of repolarisation. This has been shown for d-sotalol [204]. However, this is likely a function of reverse-use-dependence which preferentially prolongs repolarisation at slower heart rates, a condition believed to promote torsade de pointes [200]. Glibenclamide is another example of an ischaemia-selective drug with effects on repolarisation that is limited in its antiarrhythmic actions. Barrett and Walker [205] found that glibenclamide showed some ischaemia-selectivity for prolonging repolarisation in that the rate of ischaemia-induced action potential shortening was slowed, however action potentials were not prolonged beyond levels found in ischaemia, as was the case with the compounds in the present study. Furthermore, the effects of glibenclamide on action potentials were undetectable by five-minutes post-occlusion. While it is not known whether the effects of compounds in this study were lost later following occlusion, it is doubtful since this activity was associated with increased selectivity for

antiarrhythmic versus proarrhythmic effects and a trend towards increased antiarrhythmic efficacy in studies that continued for 15 minutes post-occlusion.

4.6 Study Limitations

The choice of three minutes post-occlusion as the time at which to evaluate drug effects on the action potential was made for reasons cited above. One of these reasons was that the occurrence of arrhythmias did not contaminate measures of action potential duration and upstroke at this time. Comparison of arrhythmic events following occlusion in whole animal models versus isolated hearts was reported by Curtis in a review of isolated heart techniques [194]. VF occurs earlier following occlusion, and in a higher percentage of animals, in anaesthetised rats compared to Langendorff-perfused isolated rat hearts. Peak incidence of VF (70%) *in vivo* occurs at around 8 minutes post-occlusion, whereas in isolated rat hearts peak incidence of VF (50%) occurs at around 17 minutes post-occlusion. Therefore our observations are consistent with known arrhythmia profiles in these models.

However, the above suggests that the time period at which ischaemia-selectivity was measured did not coincide with the occurrence of arrhythmias and thus may not be suitable to relate to antiarrhythmic protection against ischaemic arrhythmias. This is confounded by the fact that arrhythmias were not quantified in isolated heart experiments.

The isolated heart model was designed primarily to quantify drug effects on the cardiac action potential in normal versus ischaemic tissue, not to evaluate antiarrhythmic efficacy. Since the aim of this study is to provide insight into the development of clinically safe and effective antiarrhythmics, it was felt that use of the *in vivo* model as a measure of antiarrhythmic efficacy was optimal, as it more closely represented clinical myocardial infarction than the isolated heart model. Therefore, whether or not arrhythmias occurred to the same extent in the isolated heart

model is not particularly relevent to this study except where it relates to the degree of ischaemia produced in the occluded zone of the isolated heart.

In this respect it could be argued that since ischaemia is arrhythmogenic, one would expect a model where arrhythmias occur earlier following occlusion, and with greater incidence, to represent more severe ischaemia than a model where arrhythmias occur less often. Thus, if anything, the ischaemia produced at three minutes post-occlusion in the isolated heart underestimates that produced at three minutes *in vivo* and certainly represents less ischaemia than that produced at later time points. Therefore, ischaemia-selectivity values determined for test compounds in the isolated heart model will, if anything, be underestimated. This merely serves to enhance the significance of the positive relationships found in this study between ischaemia-selectivity and antiarrhythmic activity.

The use of potency indices such as D25% involves particular considerations. The determination of a 25% increase in response above control levels does not require knowledge of the maximum possible response obtainable, either physiologically or due to drug efficacy. It is thus useful when experimental conditions make measurements of maximal responses impossible. However, it does limit the ability to compare D25% values from different variables. To do so implies that the parameters studied did not vary much in the magnitude of their maximal responses. If they did, the D25% for effects on one parameter could be closer to the true ED_{50}, whereas for another it could be nearer to ED_{25}. In this study it was determined using non-linear regression analysis that variation in estimates of maximum response of up to 100 fold did not significantly affect determination of D25% within a parameter, and it is likely that most variables measured were limited by physiological constraints such that in reality they did not differ to an extent greater than that. It is also difficult to compare potency as determined by D25% with that determined by ED_{50}. These two values cannot be directly compared since, in the former case, it

is unknown what fraction of the total dose-response curve is represented by a 25% increase from control.

There were other limitations in this study that relate to comparisons of effects in isolated heart experiments versus arrhythmia experiments. In coronary occlusion and electrical stimulation experiments drug effects were measured relative to infused dose (μmol/kg/min). In isolated heart experiments drug effects were measured relative to concentration in the perfusate (μM). To directly compare these amounts it would be necessary to determine the concentration of drug in cardiac tissue in a whole animal for a given dose of drug. This would involve measuring plasma drug concentration, coronary flow rate, rate of absorption of drug by cardiac tissue, the rate of redistribution from the heart to other organs, and the rate of elimination of drug, among other variables. Since this information was not available and was beyond the scope of this investigation such calculations could not be performed.

The choice of a five-minute infusion regimen in coronary occlusion and electrical stimulation experiments was based on the limited pharmacokinetic data available for these compounds. The aim was to achieve a pseudo steady-state plasma concentration level. A bolus dosing regime would lead to rapid alpha-phase redistribution without a sustained plasma concentration and would likely have contributed to variation in the results. The infusion protocol was based on the assumption that the volume of distribution is small and therefore saturation of the alpha phase occurs in a shorter time span and the attainment of some peak plasma concentration will be achieved earlier (see Yong [116]). In support of the these assumptions, drug effects on electrophysiologic and hemodynamic parameters were reported as early as 3 minutes following the start of infusion (see results for electrical stimulation experiments above). Where pharmacokinetic studies have been performed with related compounds, it was found that serum concentrations rose significantly over the first 5 minutes of *in vivo* infusion (Nortran

Pharmaceuticals, personal communication). If however, plasma levels had not reach steady-state this would likely serve to overestimate potencies for various drug effects for these compounds.

Comparison of *in vivo* studies with *in vitro* studies in this study involved an important distinction: the use of anaesthesia. For coronary occlusion and electrical stimulation experiments rats were anaesthetised with sodium pentobarbital, whereas no anaesthetic agent was used in isolated hearts. Sodium pentobarbital has been shown to have effects on cardiac repolarisation. In the arterially-perfused canine wedge preparation sodium pentobarbital, in clinically relevent concentrations, dramatically reduces dispersion of repolarisation across the transmural surface of the left ventricular wall [206]. Furthermore, it inhibited the effects of d-sotalol on transmural distribution of repolarisation such that d-sotalol-induced transmural dispersion is not observed [207]. This has been used as a model of long QT syndrome. The drugs used in this study all prolonged repolarisation in cardiac action potentials and prolonged ERP, reduced MFF, and prolonged the QT1 and QT2 intervals. It is possible that if these drugs are antiarrhythmic by virtue of reducing heterogeneity of repolarisation in the ischaemic zone that such effects may be more apparent *in vitro* than *in vivo*.

The induction of arrhythmias by electrical stimulation may be due to different mechanisms than arrhythmias induced by coronary occlusion. Thus comparison of potencies for antiarrhythmic protection in ischaemic versus non-ischaemic arrhythmia models may not solely involve considerations of the presence or absence of ischaemic tissue. However, in general it is believed that electrical stimulation induced arrhythmias are due to induction of reentrant circuits as is the case with ischaemia-induced arrhythmias [208].

RSD944 was usually either at the left or right end of rank orders of potency and other variables. It therefore had an important influence on the outcome of correlation analysis. Because this drug failed non-linear regression analysis in coronary occlusion experiments, slope and ED_{50} could not be determined using regression. Instead, for the purposes of correlation analysis, it was

ranked as having the most shallow slope, and the greatest potency. This was based on visual examination of the dose-response curve.

The failure of RSD944 to fit to the regression equation with any significance was due to the fact that arrhythmia scores showed no apparent relationship with dose. This may be due to poor dose selection; the doses tested may represent the right-most portion of the dose-response curve, which would suggest that this drug has low efficacy. Barrett et al [96] showed a similar dose-response curve for this compound in a related experiment. They found no discernable dose-dependent effect for arrhythmia score and the doses used were lower than those of this study, suggesting that our findings were not due to having sampled doses that were too far to the right of the dose-response curve, but rather reflect a true lack of specificity for protection against ischaemia-induced arrhythmias. These results suggest that RSD944 inhibits ischaemia-induced arrhythmias via a non-specific, and therefore non-dose related, mechanism. This would provide an explanation for poor slope and goodness of fit variables that are consistent with lack of selectivity for antiarrhythmic effects.

A final consideration is the compounds used in this thesis. These were available only in limited quantities and this restricted the scope of the investigation. Ideally, in isolated heart studies complete dose-response curves would have been determined for effects on V_{max} and APD, but sufficient amounts of drug were not available for this. The question of chemical stability may be also raised, as for several of these compounds such information is not readily available. However, the close correlation between our results and those of previous investigators who used similar, and in some cases identical, compounds indicate that handling and storage of the compounds did not affect the results.

CONCLUSIONS

In summary, this study showed that drugs which were potent for effects in normal tissue, whether for conduction or repolarisation, were potent at protecting against non-ischaemic arrhythmias. These compounds were also potent at protecting against ischaemia-induced arrhythmias by virtue of equivalent effects in ischaemic tissue. However, they were also associated with proarrhythmic effects as indicated by poor goodness of fit and shallow slopes for antiarrhythmic dose-response curves in coronary-occlusion experiments. These proarrhythmic effects were due to a lack of ischaemia-selectivity for effects on excitability combined with small ischaemia-selectivity ratios for effects on repolarisation.

On the other hand, drugs that showed less potency for effects in normal tissue were less potent at protecting against non-ischaemic arrhythmias, and appeared to do so via prolongation of repolarisation since potency for these effects far exceeded that for effects on excitability. The relative lack of potency for conduction and repolarisation effects may explain their lack of potency for protection against ischaemic arrhythmias as well. However, these compounds were considerably more potent at prolonging repolarisation in ischaemic tissue relative to normal tissue, and this explains their steep slopes and strong goodness of fit values. Thus proarrhythmic effects in these compounds were limited due to lack of potency for electrophysiologic effects in normal tissue.

The results of this thesis support the central hypothesis that in this series of compounds superior antiarrhythmic protection is a consequence of ischaemia-selective electrophysiological actions. This work suggests a new and positive direction in which to guide antiarrhythmic drug development.

APPENDICES

Appendix A

Calculations for Making Drug Solutions (Electrical Stimulation Experiments)

The design of this experiment was random and blind. Five rats were used in each drug treatment group and five rats for controls (non-treated) yielding a total of 35 rats. The amount of drug pre-weighed into vials before randomization was determined using the following equation (control vials were left empty until saline was added, as below):

$$\textbf{amount weighed out} = \frac{\textbf{starting dose of drug (µmol/kg/min) x rat weight (kg) x solution volume (mL)}}{\textbf{starting infusion rate (mL/min)}}$$

{Equation 7}

Amounts were based on a 300 g rat with a solution volume of 6 mL and a starting infusion rate of 0.5 mL/hour:

$$\textbf{amount weighed out} = \frac{\textbf{starting dose of drug (µmol/kg/min) x 0.3 kg x 6 mL}}{\textbf{0.00833 mL/min}}$$

{Equation 8}

For each rat the amount of saline in which to dissolve the drug was calculated using the following formula:

$$\textbf{amount of saline to add} = \textbf{300 kg/rat weight (kg) x 6 mL}$$

{Equation 9}

Appendix B.

Results of ANOVA for Coronary Occlusion Experiments

H_o= null hypothesis: groups represent samples of the same population

$F_{critical} = F_{0.05(1),6,28} = 2.45$

RSD1031

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	4.495709	6	0.749285	20.13124	5.7E-09	**2.45**
Within Groups	1.04216	28	0.03722			
Total	5.537869	34				

Conclusion: reject H_o

RSD1030

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	4.03846	6	0.67307	10.528926	4.057E-06	**2.45**
Within Groups	1.78994	28	0.06392			
Total	5.82840	34				

Conclusion: reject Ho

RSD1020

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	4.433643	6	0.738941	7.684982	6.08E-05	**2.45**
Within Groups	2.692308	28	0.096154			
Total	7.125951	34				

Conclusion: reject Ho

RSD995

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	4.228354	6	0.704726	6.836971	0.000152	**2.45**
Within Groups	2.88612	28	0.103076			
Total	7.114474	34				

Conclusion: reject Ho

RSD988

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	1.727183	6	0.2878638	4.192952	0.004438	**2.47**
Within Groups	1.78501	28	0.0686542			
Total	3.512193	34				

Conclusion: reject Ho

RSD944

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	2.1132713	6	0.3522119	3.31675	0.0135626	**2.45**
Within Groups	2.9733728	28	0.1061919			
Total	5.0866441	34				

Conclusion: reject Ho

Appendix C:

Isolated Heart Experiments: Raw Data for Control and Sham Occlusions

Upstroke Velocity: Each value in column **A** and **B** is the first derivitive of the action potential, $d(\Delta F/F)/dt_{max}$; column **C** is the percent change from control, ΔV_{max}

CONTROL OCCLUSIONS		
Normal tissue		
A. Pre-occlusion	**B.** 3' post-occlusion	**C.** [(A – B)/A]*100
11446	8357	26.988
10399	7012	32.57
9988	8055	19.353
7484	6496	13.201
6700	5174	22.776

Mean ± S.E.M. = 23 ± 3.0

CONTROL OCCLUSIONS		
Ischaemic tissue		
A. Pre-occlusion	**B.** 3' post-occlusion	**C.** [(A – B)/A]*100
27390	11220	26.988
33708	11206	32.57
24587	14100	19.353
20001	9803	13.201
14990	5583	22.776

Mean ± S.E.M. = 56 ± 3.0

SHAM OCCLUSIONS		
Normal tissue		
A. Pre-occlusion	**B.** 3' post-occlusion	**C.** [(A – B)/A]*100
11059	7785	29.605
7634	6377	16.466
6943	4792	30.981
6834	5387	21.174
7812	6130	21.531

Mean ± S.E.M. = 24 ± 3.0

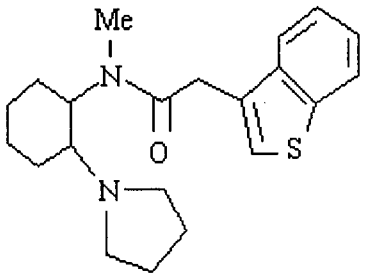
SHAM OCCLUSIONS		
Normal tissue		
A. Pre-occlusion	**B.** 3' post-occlusion	**C.** [(A – B)/A]*100
10886	8293	23.82
7494	6511	13.117
12535	8137	35.086
15215	13220	13.112
7477	5249	29.798

Mean ± S.E.M. = 23 ± 4.0

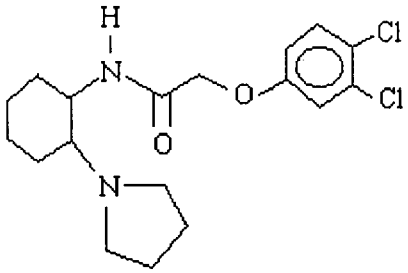
Appendix D:

Structures of RSD compounds used in this thesis:

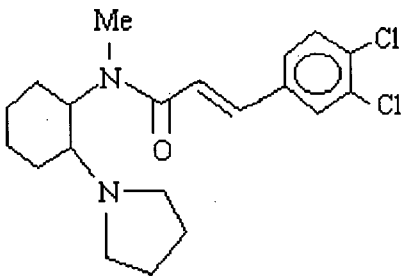
RSD944



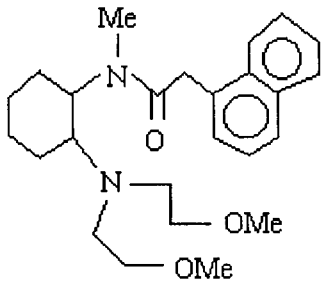
RSD988



RSD995



RSD1020



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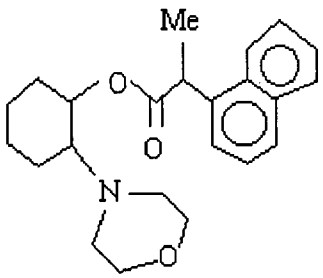
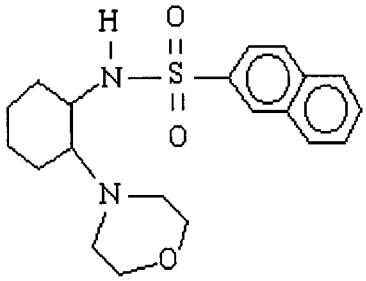


RSD1031



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