The electrophysiological effects of Endothelin-1 in human atrial myocytes.

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This thesis is being submitted for the degree of Ph.D. at the University of Glasgow

This work was carried out in the Section of Cardiology (Royal Infirmary), Division of Cardiovascular & Medical Sciences at The University of Glasgow.

Abstract

Introduction: Chronic heart failure (CHF) is associated with an increased incidence of atrial fibrillation (AF) and elevated levels of catecholamines and endothelin-1 (ET-1), each of which affects the atrial L-type calcium current (I_{CaL}) and consequently action potentials.

Hypotheses: ET-1 modulates the effects of isoproterenol (ISO) on I_{CaL} and action potentials in human atrial myocytes.

Methods: Atrial myocytes were isolated enzymatically from samples of right atrial appendage obtained from consenting patients in sinus rhythm undergoing cardiac surgery. The nystatin-perforated whole cell patch clamp technique was used at 37ºC to record I_{CaL} and action potentials in voltage-clamp and current-clamp mode respectively.

Results: The current-voltage relationship of I_{CaL} was bell-shaped, peaking at +10 mV with a current density of -4.8±0.4 pA/pF (mean± s.e.m., n=89 cells, 34 patients). ISO, 0.1 nM to 1 μM, increased peak I_{CaL} in a concentration-dependent manner (n=4-46 cells) with a maximum response of 250± 53% above control and an approximate EC_{50} of 0.06 μM. Isoproterenol at 0.05 μM significantly increased peak I_{CaL} from -4.7± 0.4 to -12.2± 0.9 pA/pF ($P<0.05$, Students *t*-test; n=64 cells). This adrenergic effect was reversed by ET-1 at all concentrations tested from 0.01 to 10 nM and was partially reversible upon ET-1 washout and in the presence of the specific $ET\text{-}_A$ receptor antagonist, FR139317 (n=5-12 cells). Neither ET-1 alone nor the $ET\text{-}_B$ receptor agonist Sarafotoxin S6c, at 10 nM, had an effect on I_{CaL}.

Isoproterenol (0.05 μM) prolonged the action potential duration at 50% repolarisation (APD_{50}) from 30± 7 to 46± 7 ms ($P< 0.05$, n=15 cells), but had no effect on APD_{90} nor the cellular ERP. These adrenergic effects on APD_{50} and SDs were also abolished by ET-1 at 10 nM ($P< 0.05$, n=15 cells). Superfusion with ET-1 (10 nM) alone had no significant effect on APD_{50}, APD_{90}, nor ERP (n=21 cells). There were no significant interactions between these electrophysiological effects and diseases states or chronic pre-operative drug therapy.

Spontaneous activity, defined as a depolarisation occurring during phase 3 of action potential repolarisation or a depolarisation of greater than 3 mV amplitude during phase 4, frequently interrupted action potential recordings during, but not prior to, superfusion with ISO. Using a repetitive stimulation protocol, ISO at 0.05 μM produced spontaneous depolarisations in 5 of 7 cells studied ($P< 0.05$, $\chi 2$ test). Endothelin-1 at 10 nM abolished these depolarisations in all 5 cells ($P< 0.05$). Superfusion with ET-1 (10 nM) alone was associated with spontaneous depolarisations in significantly fewer cells ($P< 0.05$, n=2 of 13 cells).

In a retrospective univariate analysis, patient comorbidity and pre-operative drug therapy were not found to influence the electrophysiological effects observed.

Conclusions: ET-1 reversed adrenergically induced increases in peak I_{CaL}, APD_{50} and SDs in human atrial myocytes. This anti-adrenergic effect may be expected to influence the occurrence of AF in patients irrespective of comorbidity or pre-operative drug therapy.

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CJ Redpath, AJ Workman, KA Kane, AC Rankin. Anti-adrenergic effect of endothelin-1 on L-type calcium current in isolated human atrial myocytes. Heart 89: 162 Suppl. 2003.

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Antony J Workman, Davide Pau, **Calum J Redpath**, Gillian E Marshall, Julie A Russell, Kathleen A Kane, John Norrie, Andrew C Rankin. Post operative atrial fibrillation is influenced by beta blocker therapy but not atrial cellular electrophysiology. Journal of Cardiovascular Electrophysiology 2006 Nov; 17(11): 1230-8

Antony J Workman, Davide Pau, **Calum J Redpath**, Gillian E Marshall, Julie A Russell, Kathleen A Kane, John Norrie, Andrew C Rankin. Human atrial cellular electrical remodelling by ventricular systolic dysfunction may predispose to AF. Heart Rhythm 2009 April; 6(4): 445-451.

Acknowledgement

I would like to express my appreciation to my Supervisors; Antony Workman, Kathleen Kane and Andrew Rankin; Head of Department, Stuart Cobbe and The British Heart Foundation, without whom this work could not have been undertaken. Additionally, I wish to thank my mother, Joyce, and especially my wife, Stephanie, without their support I would not have been in a position to consider undertaking this work in the first instance.

I would like to acknowledge the co-operation of the surgical staff at The Royal Infirmary of Glasgow and Julie Russell who was always helpful in the laboratory.

Above all I wish to commend the generosity and fortitude of the patients, who, in their time of need, were overwhelmingly prepared to help others.

I am very grateful to you all.

Author's declaration

The database of patients' clinical characteristics was designed by Antony Workman and maintained by Julie Russell. All other work presented herein is entirely the author's own.

Abbreviations

α_1-AR	alpha type 1 adrenoceptor
α_2-AR	alpha type 2 adrenoceptor
β_1-AR	beta type 1 adrenoceptor
β_2-AR	beta type 2 adrenoceptor
ACE-I	angiotensin converting enzyme inhibitor
AF	atrial fibrillation
AF_{pcs}	atrial fibrillation post cardiac surgery
A-II	angiotensin II
AMP	adenosine monophosphate
APD_x	action potential duration (x% repolarisation)
ATP	adenosine triphosphate
AVN	atrio-ventricular node
AVR	aortic valve replacement
β-B	beta-adrenoceptor antagonist (beta-blocker)
bpm	beats per minute
C	capacitance
CABG	coronary artery bypass graft
CCB	calcium channel antagonist (blocker)
cAMP	cyclic adenosine monophosphate
cSEVC	continuous single electrode voltage clamp
CHF	chronic heart failure
DAD	delayed afterdepolarisation
DMSO	dimethylsulphoxide
E	epinephrine
E_m	resting membrane potential
ECG	12 lead electrocardiogram

EAD	early afterdepolarisation
ECE	endothelin converting enzyme
ERP	effective refractory period
ET-1	endothelin-1
ET-2	endothelin-2
ET-3	endothelin-3
$ET\text{-}_A$	endothelin receptor type A
$ET\text{-}_B$	endothelin receptor type B
G_i	G protein inhibitory subunit
G_s	G protein stimulatory subunit
GDP	guanosine diphosphate
GTP	guanosine triphosphate
GPCR	guanyl-nucleotide regulatory protein complex receptor
HBP	hypertension
HR	heart rate
I	current
I:V	current: voltage relationship
I_{CaCl}	calcium activated chloride current
I_{CaL}	L-type calcium current
I_{ClcAMP}	cyclic AMP dependent Chloride current
I_f	funny current
I_{Kur}	ultrarapid delayed rectifier current
I_{K1}	inward potassium current
I_{NaCa}	sodium calcium exchange current
$I_{Na/H}$	sodium hydrogen exchange current
I_{ti}	transient inward current
ICM	ischaemic cardiomyopathy

IP_3	inositol tri-phosphate
ISO	isoproterenol
KB	kraftbrüe
LJP	liquid junction potential
LtP	leak to peak current
LVSD	left ventricular systolic dysfunction
MI	myocardial infarction
mRNA	messenger ribonucleic acid
n_c	number of cells
n_p	number of patients
NE	norepinephrine
NICM	non-ischaemic cardiomyopathy
NO	nitrous oxide
NP	nystatin perforated electrical access
NR	nystatin ruptured electrical access
pCLAMP	Axopatch amplifier analysis software
PKA	protein kinase A
PKC	protein kinase C
PLC	phospholipase C
PDE	phosphodiesterase
R	resistance
REP	repetitive stimulation protocol
R_p	pipette resistance
R_s	series resistance
SD	spontaneous depolarisations
S-S6c	sarafatoxin S-6c
SR	sarcoplasmic reticulum

tau_f	time constant (fast)
tau_s	time constant (slow)
TCC	time course constant protocol
TTE	trans-thoracic echocardiography
WinWCP	windows whole cell patch clamp software.

1 GENERAL INTRODUCTION

1.1 Atrial Fibrillation- the clinical problem

Atrial Fibrillation (AF) is the most common sustained cardiac arrhythmia in humans and epidemiological studies have demonstrated that the incidence of AF is increasing[1]. Characterised by multiple electrical wavelets spreading chaotically throughout the atrium resulting in rapid irregular stimulation of the ventricles via the atrioventricular node (AVN), AF causes an irregular tachycardia. Ventricular contraction in AF is both irregular in frequency and force of contraction as a result of the Frank-Starling mechanism[2]. This may produce an unpleasant sensation of palpitation, and in addition, the tachycardia, if prolonged, results in atrial contractile dysfunction, a pro-thrombotic state predisposing to thrombo-embolic disease and a reduction in cardiac output which can precipitate heart failure[3;4]. Patients who develop AF suffer increased morbidity and an approximate doubling of mortality compared with aged-matched controls[5]. Treatment has traditionally aimed at either terminating the arrhythmia by cardioverting the patient back to sinus rhythm, 'rhythm control', or slowing the ventricular rate in AF via drugs which increase the refractory period of the AVN, 'rate control'. Recent evidence has demonstrated that the increased morbidity and mortality in AF is maintained in both rhythm and rate control strategies due to the combination of anti-arrhythmic drug toxicity, insufficient anti-coagulation to prevent thromboembolism and the high relapse rate of AF[6-8].

1.2 Fundamental mechanisms of arrhythmogenesis in human atria

The three fundamental mechanisms underpinning arrhythmogenesis are triggered activity, reentry and abnormal automaticity the occurrence of which is promoted by several pathophysiological remodelling processes (Figures 1.1-1.4). In order to understand any potential effects on human atrial arrhythmogenesis a brief description of the ionic flux during a normal human atrial action potential will be followed by a discussion of each of the three fundamental mechanisms in turn.

Under normal circumstances the rapid upstroke of the human atrial myocyte action potential (phase 0), mediated by I_{Na}, occurs as an all or none response if the cell is depolarised to threshold (-50 mV) by its neighbour from the resting potential of approximately -75 mV. This rapid upstroke reaches positive potentials (+40 to +60 mV) and is immediately followed by transient repolarisation comprised of rapidly inactivating fast sodium current (I_{Na}) and activation of the repolarising transient outward and ultra-rapid rectifying potassium currents I_{to} and I_{Kur} (phase 1)[9]. Membrane depolarisation (above -40 mV to peak activation at +10 mV) activates L-type calcium current (I_{CaL}) which maintains the action potential plateau (phase 2) providing the electrochemical driving force for K^+ efflux, and as I_{CaL} inactivates, the rapid and delayed rectifier repolarising currents I_{Kur}, I_{Kr}, I_{Ks} and I_{KATP} predominate returning the membrane voltage back towards the resting potential (phase 3)[9]. The atrial myocyte is relatively quiescent during phase 4, in readiness for subsequent stimulation (Figure 1.4).

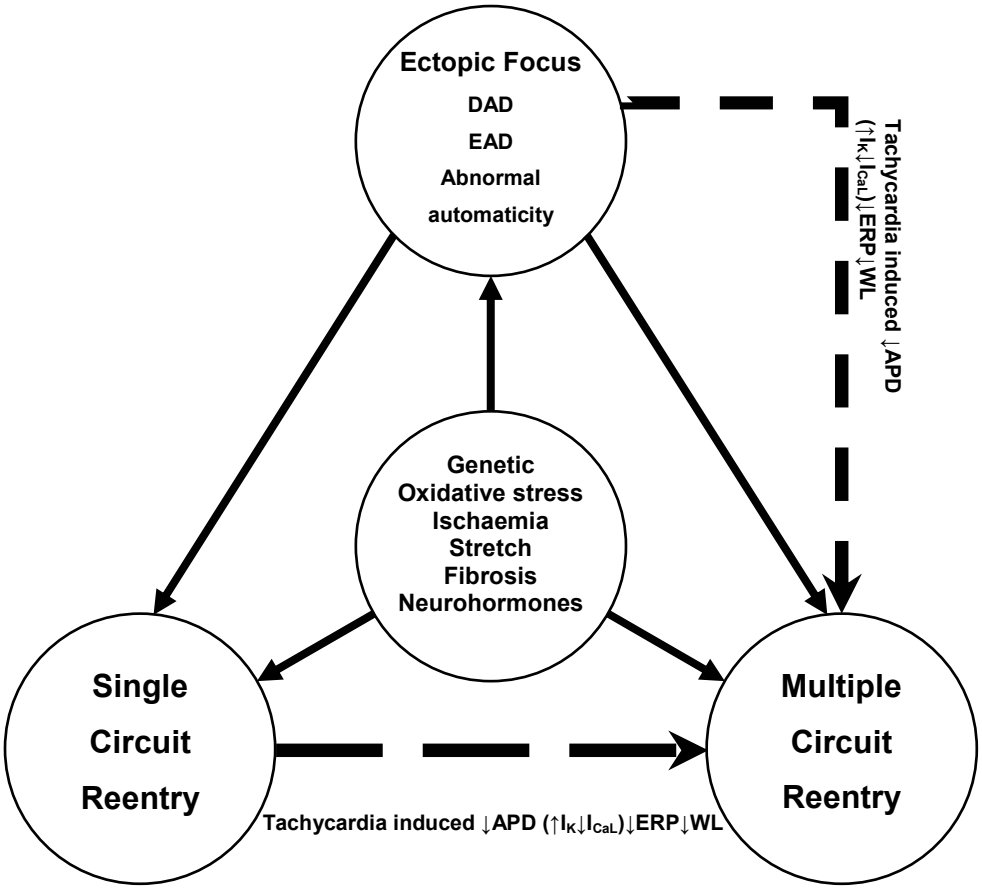


Figure 1.1 Schema indicating the complex inter-relationships between the fundamental mechanisms of arrhythmogenesis, human atrial electrophysiological remodelling and atrial pathophysiologies known to be associated with AF in humans.

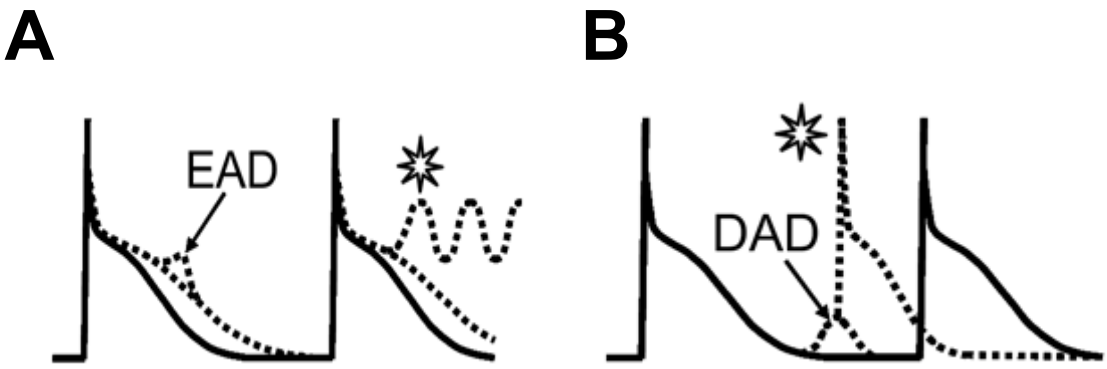


Figure 1.2 Representations of A) EADs and B) DAD in human atrial myocytes adapted from Workman et al, Heart Rhythm 2008;5:S1-S6. Typical human atrial action potentials in bold with shaded lines indicating the impact of early or late depolarisations on action potential morphology and subsequent generation of triggered activity, indicated by star symbol.

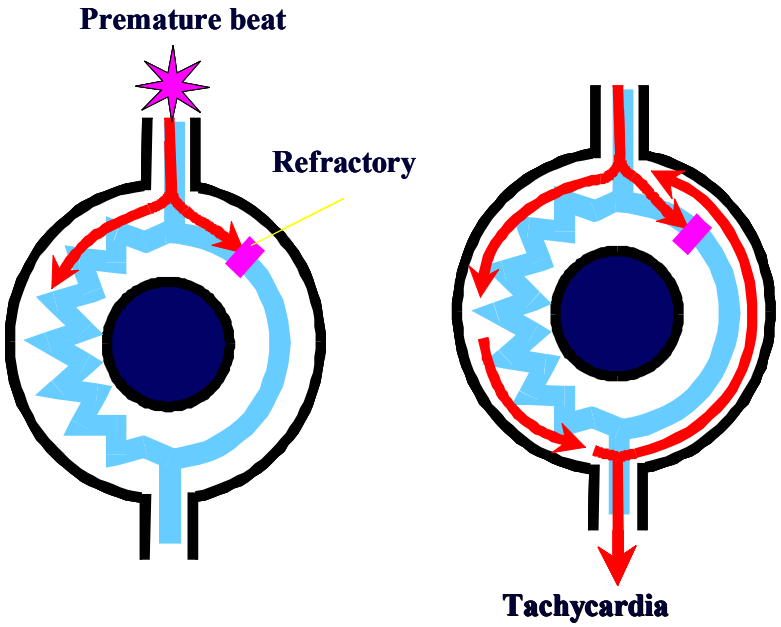


Figure 1.3 Schema displaying the formation of a 2 dimensional reentrant circuit.

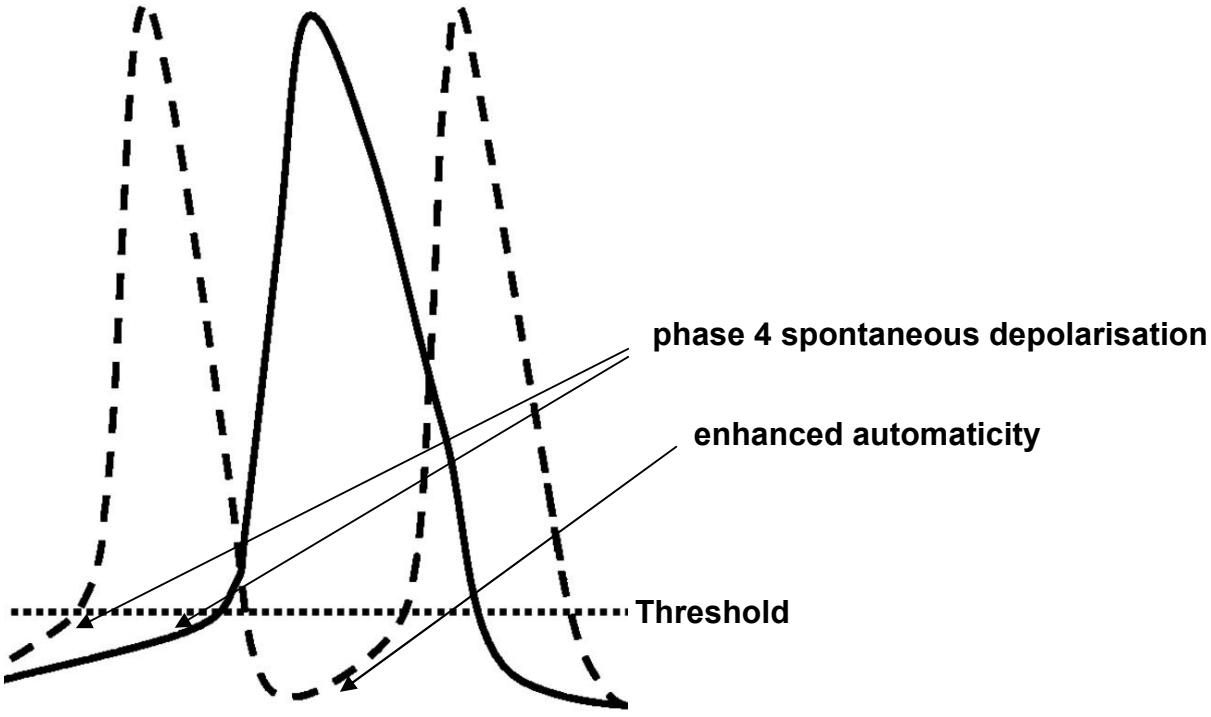


Figure 1.4 Schema displaying abnormal automaticity due to accelerated depolarisation of phase 4 of a stylised action potential.

1.2.1 Triggered Activity

Triggered activity, by definition, are abnormal afterdepolarisations dependent on the preceding action potential for their generation which, if reaching threshold, trigger a subsequent action potential. These diastolic oscillations in the membrane potential are classified according to the phase of the preceding action potential during which they occur (Figure 1.2).

Early depolarisations (EADs), which by definition disrupt repolarisation in phase 2 or 3 of the action potential, are associated with prolongation of repolarisation and bradycardia or pause dependent mechanisms[10]. Delayed afterdepolarisations (DADs), however, which occur during phase 4 of the action potential are often associated with tachycardia. In the presence of β- adrenergic stimulation both EADs and DADs may occur at physiological heart rates due to cellular calcium loading albeit by differing mechanisms[10;11]. If an EAD is sufficiently large it can cause the cell to reach threshold and result in a new subsequent action potential manifesting as an ectopic beat on the surface electrocardiogram (ECG) or, as a result of electrotonic interaction with surrounding myocardial cells in various stages of refractoriness, can result in initiation of a reentrant arrhythmia[12;13]. Triggered activity has been almost exclusively studied in ventricular preparations, although occasional atrial experiments have been reported[14-16].

EADs are associated with a reduction in rapid and slow activating delayed rectifier currents I_{Kr} and I_{Ks} combined with augmentation of I_{CaL} and late I_{Na} which prolongs the APD sufficiently to allow I_{CaL} to recover from inactivation and reactivate during the plateau of the action potential[10;11;17-20]. EADs have consistently been induced by stimulation of beta-adrenoceptors (β-AR) by ISO[21;22] resulting in aftercontractions[22;23]. EADs were initially thought to be particular to the conducting

tissue or Purkinje network[24] but more recently have been demonstrated in atrial preparations although their clinical relevance remains to be determined[15;20;25].

DADs are a result of spontaneous SR calcium release at the end of the action potential which activates the transient inward current (I_{ti}) comprised primarily of the sodium-calcium exchange current (I_{NaCa}) and the calcium activated chloride current ($I_{Ca(Cl)}$) under conditions that favour cellular calcium overload[11;16;20;26-29]. Under normal circumstances calcium entry via I_{CaL} and reverse mode I_{NaCa} acts as the trigger for calcium induced calcium release (CICR) from the sarcoplasmic reticulum (SR) resulting in a calcium transient[30-33]. The local control theory[34] linking the close proximity of the I_{CaL} pores with the ryanodine receptors of the SR in the dyadic cleft explains how calcium sparks can be graded and both temporally and spatially summated into global calcium transients depending on the magnitude of the whole cell I_{CaL}, SR calcium content and ryanodine receptor status[35-43]. The direction and magnitude of I_{NaCa} is dependent on the myocyte membrane potential and the local concentration gradients across the plasmalemma of Ca^{2+} and Na^{+} and the stoichiometry of the reverse mode I_{NaCa} results in a net inward current prolonging myocyte repolarisation and APD[44].

Confocal microscopy has demonstrated differing spatial features of EADs as compared with DADs[45], with greater homogeneity demonstrated following an EAD, as expected with membrane bound electrophysiological mechanisms, as opposed to the focal spontaneous SR calcium release resulting in a DAD, although significant overlap may exist[10;22;45-47]. Indeed recent evidence has identified a potential role for I_{NaCa} in EAD formation both as conditioning agent and as charge carrier[10;11;17;48;49].

ET-1 was, at first, thought likely to predispose to EADs and DADs by reducing I_K and causing increased intracellular calcium loading[50] via augmenting I_{CaL}. The Pulmonary Vein sleeves, thought by many to be the predominant driver behind most AF[51], may be particularly prone to triggered arrhythmia[52;53]. Although results from initial experiments investigating potential arrhythmogenic effects of ET-1 in cardiac tissue remain difficult to interperet[54-60], several groups reported focal 'arrhythmia' or spontaneous contractions following acute exposure to ET-1[56;61-64]. The potential for ET-1 to have arrhythmogenic effects in human atrium remains to be determined.

1.2.2 Intra-atrial Reentry

Intra-atrial reentry occurs when a depolarising wavefront being propagated between atrial myocytes encounters an area of unidirectional block. If the aetiology of block is functional, should the wavefront travel in another direction and return to this refractory tissue when it is no longer refractory it will now conduct creating a circuit (2 dimensional circuit represented in Figure 1.3). Conduction within the circuit will be maintained if in the intervening period neighbouring myocytes have regained excitability. If the wavefront travels along the entire circuit (Conduction Time) sufficiently slowly (Conduction Velocity) that all points within the circuit (Path Length) recover from refractoriness the circuit may be maintained indefinitely. The wavelength is the product of refractory period and conduction velocity and as such it represents the distance traveled by an impulse in one refractory period. The wavelength determines the shortest circuit that may accommodate functional reentry. Thus any factor that reduces conduction velocity and refractoriness or increases path length or conduction time will promote reentry and ultimately AF. Decreased I_{Na} and upstroke velocity (V_{max}), decreased I_{CaL} or increased I_K,

decreased action potential duration (APD) and effective refractory period (ERP), atrial dilation and stretch all promote reentry.

Traditionally considered in terms of the "leading-circle model", as illustrated in Figure 1.3, recent developments in biophysical theory have suggested the utility of a "spiral wave model" of reentry[65;66]. In cardiac tissue, a spiral wave of reentry can occur following an ectopic beat, howsoever generated, that initiates a depolarising wavefront which traverses the trajectory of a previous sinus beat and encounters tissue in various stages of repolarisation. This second wavefront blocks in refractory tissue, however is able to propagate across non refractory tissue, albeit tangentially in 3-dimensions, curving towards newly excitable myocytes[65]. The major difference between the leading-circle and spiral wave models of reentry, other than level of complexity, is the improved correlation of the impact of I_{Na} blockade on re-entrant circuit characteristics predicted by the spiral wave model.

1.2.3 Abnormal Automaticity

Abnormal automaticity in the atrium occurs when atrial or pulmonary vein myocytes, which are not normally pacemaker cells, begin to spontaneously fire as a result of accelerated phase 4 depolarisation (See Figure 1.4). If the rate at which these cells reach threshold exceeds that of the sino-atrial node (SAN), then an ectopic rhythm will supervene. This form of regular tachycardia may act as a trigger to atrial tissue that is vulnerable to reentry which may fractionate, or may result in fibrillatory conduction because the atrial myocardium is not capable of maintaining 1:1 conduction for some reason (see below). Although the primary phase 4 depolarising current, I_f, is expressed in atrial tissue from patients suffering AF and in porcine CHF models no direct evidence for a role of abnormal automaticity has been identified to date in human patients[67-69].

1.3 AF begets AF: electrophysiological remodelling

It has long been established that the longer the duration of AF the lower the likeliehood of successful cardioversion and subsequent maintainance of sinus rhythm indicating that the arrhythmia itself is associated with changes which result in perpetuation of the arrhythmia[70;71]. More recently in animal models using rapid atrial pacing to induce AF and in observational clinical studies, atrial electrophysiological and cellular changes have been shown to rapidly occur favouring the arrhythmia becoming permanent[72-74]. The electrical changes, collectively termed electrophysiological remodelling, result in a shortened atrial action potential duration (APD) and an abbreviated atrial effective refractory period (ERP)[73]. In addition the normal physiological rate adaptation of human atrial APD and ERP which results in proportional shortening of the APD and ERP with increasing heart rate is lost[73]. Combined with decreases in conduction velocity[75-77] and increased heterogeneity in atrial repolarisation[78] these AF induced changes in APD and ERP create a substrate for the multiple wavelet circuit re-entry necessary to perpetuate AF.

Isolated human atrial myocyte electrophysiology experiments corroborate these changes in APD and ERP and indicate that the ionic mechanisms which underpin electrophysiological remodelling can be attributed, at least in part, to alterations in the L-type calcium current (I_{CaL})[79;80]. Reductions in human atrial myocyte peak I_{CaL} current density have been associated with chronic atrial fibrillation in a number of studies[81-84] ranging from 50% (Christ), 63% (Workman) to 75% (Skasa) in the presence (Christ) and absence (Workman) of a change in current voltage relationship[79] and have been attributed to various protein expression downregulation of the pore forming α_{-1c} subunit[85-87], although this remains controversial[88;89]. More recently the interaction of intracellular kinases and

phosphatases have been implicated[84;90-92]. These observations only partly explain AF induced remodelling however.

1.4 AF begets AF: cellular remodelling

The multiple re-entry circuits of AF produce very high frequency electrical activity in the atrium *in vivo*, typically up to 600 beats per minute[93]. Rapidly increasing rates of electrical stimulation of human atrial myocytes *in vitro* are associated with large increases in calcium influx contributing to calcium overload[94]. In chronic rapid atrial pacing models of AF, calcium overload has been implicated in alterations in atrial myocyte subcellular apparatus; sarcoplasmic reticulum disruption, mitochondrial swelling[74;95], dedifferentiation and hypertrophy resulting in apoptotic cell death[96]. Similar ultrastructural changes have been described in human atrial myocytes from patients in AF[97]. During the first few days of AF the time course of the development of atrial contractile dysfunction parallels that of electrical remodelling[98], suggesting that these two processes share similar or common mechanisms. However the offset kinetics are different in that although electrical remodelling reverses within days, atrial mechanical dysfunction persists for some weeks[99]. Down regulation of synthesis of calcium handling and I_{CaL} channel proteins have been reported[85;100-102] in response to calcium overload. The time course of mRNA regulation and ultimately expression of calcium handling apparatus is in the order of 3 months, many times longer than that of electrophysiological remodelling[100;102]. Experimental evidence supports the pivotal role of I_{CaL} in these maladaptive processes. The calcium channel blocking drug verapamil reduced both electrical remodelling and contractile dysfunction while, in contrast, calcium loading of the cell with the dihydropyridine agonist BAY 8644 had the opposite effect[103;104]. The administration of intracellular calcium lowering

agents to patients has been demonstrated to reduce the AF recurrence rate following successful DC cardioversion[105].

1.5 Atrial Fibrillation and Chronic Heart Failure

AF does occur in the absence of structural heart disease, however it is more commonly associated with cardiac disease, particularly chronic heart failure (CHF). Observational studies have associated CHF with an approximate six fold increase in the development of AF, and AF is positively correlated with the severity of CHF[5;106]. The incidence of AF and CHF doubles for every successive decade of age and is estimated to increase, in part, due to an ageing population[1;107-110]. In patients with CHF the development of AF is associated with a further deterioration in morbidity and mortality[4]. Atrial Fibrillation induced atrial contractile dysfunction combined with the irregular tachycardia immediately compromises ventricular filling and if the tachycardia persists can lead to dilated cardiomyopathy[111].

1.6 CHF induced atrial electrophysiological and structural remodelling

Several possible contributing pathophysiological mechanisms link AF and CHF. Rapid ventricular pacing induced CHF in dogs and was associated with electrophysiological remodelling in atrial myocytes[112;113]. These ventricular-paced CHF-induced reductions in atrial I_{CaL} (~30%) are smaller than those reported in rapid atrial pacing induced ionic remodelling (~70%)[113]. However in human atrial myocytes from patients with CHF reductions in I_{CaL} (~80%)[114] are similar in magnitude to those associated with AF (~65%)[79;81]. CHF causes elevated filling pressures resulting in haemodynamic stretch producing atrial dilatation which is also associated with diminished atrial I_{CaL}[115] and the development of AF[116].

Histological examination of atrial myocytes from patients with CHF demonstrated myocyte hypertrophy with evidence of local atrial conduction slowing, interstitial fibrosis and apoptosis, creating a suitable substrate for arrhythmia[117;118]. Such changes in the atria of animals with heart failure have been shown to be effective substrates of anisotropic and/ or very slow conduction which could permit the development of multiple circuit re-entry even within very small areas of the atrium[119]. These results demonstrate that the electrophysiological changes associated with AF/ rapid atrial pacing and CHF share the common mechanism of I_{CaL} remodelling.

1.7 CHF induced neurohormonal activation

The publication of a large number of negative results from trials examining haemodynamic interventions in heart failure stimulated investigation of alternative pathophysiological mechanisms in CHF. The neurohormonal hypothesis of CHF[120;121] postulated that a number of neurohormones such as catecholamines, Angiotensin II (A-II)[122] and Endothelin-1[123;124] are elevated acutely during myocardial embarrassment in order to preserve cardiac output and maintain vital organ perfusion. Initially beneficial, if the reduction in cardiac output is irreversible, as in CHF, the ensuing ventricular dysfunction produces persistent atrial stretch, baroreceptor dysfunction, excessive adrenergic activation, neurohormonal activation[125] and a positive feedback cycle resulting in a progressive deterioration in cardiac function. The ability of the failing ventricle to respond to endogenous or exogenous catecholamines becomes markedly attenuated[126] and a new cardiovascular equilibrium is reached in which peripheral vasoconstriction and fluid overload supervenes. Perfusion of the other vital organs, most notably the kidney, is adversely affected resulting in yet further sympathetic activation until cardiac output is insufficient to maintain life.

Elevation in the serum concentrations of catecholamines and endothelin-1 are positively correlated with deteriorating left ventricular systolic function, regardless of aetiology[122;125;127] and are associated with poor outcome in patients with CHF[122;128;129]. In patients with CHF, elevated serum levels of the endothelin precursor big ET-1 are independently predictive of the development of AF[130], and the onset of AF in these patients is associated with yet further elevation of serum levels of ET-1[131] and a poorer prognosis[4]. Interruption of the neurohormonal positive feedback cycle by beta-blockers (β-B)[132;133], formerly contraindicated in patients with CHF due to their adverse haemodynamic impact, are now of proven mortality benefit in patients with CHF[134-137] and thus are now recommended therapy[138]. Atrial Fibrillation commonly complicates CHF, indeed often causing haemodynamic decompensation[1;139;140]. There is also increasing evidence that beta-blockers have a role in both 'rhythm control'[141-143] and 'rate control' treatment strategies and this is supported by mechanistic evidence for an adaptive atrial cellular electrophysiological response to chronic beta blockade in patients termed ‘pharmacological remodelling’[144].

1.8 Endogenous and exogenous catecholamines

The endogenous catecholamines, dopamine, norepinephrine (NE) and epinephrine (E) are sympathomimetic amines derived from L-tyrosine which act as neurotransmitters throughout the nervous system in humans. During periods of physiological stress the adrenal medulla acts as a neuroendocrine transducer, transforming efferent impulses from the pre-ganglionic sympathetic fibres into hormonal signals, releasing NE and E into the peripheral blood to mediate the “fight or flight” response. Under normal conditions the neurohormones E and NE are both detectable in low concentration in peripheral blood. However in CHF patients the concentrations of catecholamines in peripheral blood are chronically

elevated and correlate with CHF severity[122]. Catecholamines acting as neurohormonal first messengers bind to membrane bound alpha (α-AR) and beta (β-AR) adrenoceptors throughout the body. These two types of adrenoceptors can be differentiated by their sensitivity to agonists. α-AR are most sensitive to NE; β-AR are most sensitive to the synthetic sympathomimetic amine isoproterenol (ISO) and the response to epinephrine is intermediate. Isoproterenol is the isopropyl analogue of E and acts almost exclusively at β-AR. β-AR are further classified into two types, β_1-AR and β_2-AR, both of which are expressed on the sarcolemmae of human atrial myocytes in the ratio of (β_1-AR 4:1 β_2-AR)[145] and both employ cAMP as a second messenger[146].

The binding of ISO with either of the β-ARs produces conformational change in the cytosolic membrane bound guanyl-nucleotide regulatory protein (G-protein) complex[147]. In human atrial myocytes there are two types of heterotrimeric G proteins associated with the β-AR: G_s and G_i, which stimulate and inhibit adenylylcyclase catalysing the formation of cyclic AMP (cAMP) from adenosine triphosphate (ATP), respectively. This enzyme cascade is further extended by the action of cAMP to activate protein kinase A (PKA), which phosphorylates intracellular effector proteins and this is counter-balanced by cellular phosphatases and phosphodiesterases (PDE) which degrade cAMP.

1.9 The positive inotropic effect of Isoproterenol

The force frequency relationship is similar in human atrium and ventricle, however, important differences in atrial microarchitecture[148] and intracellular calcium processing exist[149;150]. In isolated strips of human ventricle selective stimulation of β_2-AR results in at most 50% of the maximal positive inotropic effect[151], whereas selective stimulation of β_2-AR in human atrial myocardium results in a maximal

inotropic response[152]. Isoproterenol acting via both β_1-AR and β_2-AR has a positive inotropic effect[153], maximal in micromolar concentrations, mediated by the cAMP second messenger system[146;154]. ISO induces an increase in intracellular calcium, both cytosolic via phosphorylation of I_{CaL}[155] and within the sarcoplasmic reticulum (SR) via PKA dependent phosphorylation of phospholamban[156].

This ISO-induced positive inotropic effect can be modified by a range of physiological, pathophysiological and therapeutic processes. Increased senescence was associated with impaired responsiveness to ISO in the absence of altered receptor expression and a combination of reduced catalytic activity of adenylate cyclase and increased G_i in human atria[157] in contrast to human ventricular myocytes in which reduced β_1-AR and G_s expression were demonstrated[158]. In atrial tissue obtained from patients with CHF the maximal positive inotropic effect of ISO was preserved despite reduced levels of beta adrenoceptors expression[158;159] in contrast to human ventricular papillary muscle in which the positive inotropic effect of ISO was blunted in association with reduced beta adrenoceptor expression[159]. The positive inotropic effect of catecholamines is greater in atrial tissue obtained from patients receiving selective β_1-AR antagonists[152] despite a lack of effect on β_2-AR density or affinity, due to enhanced β_2-AR G_s-protein coupling[160].

1.10 The effect of ISO on I_{CaL} and action potentials

Influx of calcium ions via I_{CaL} depolarises the atrial myocyte contributing to the action potential plateau[161] and stimulates calcium-dependent-calcium-release from the sarcoplasmic reticulum thereby permitting excitation:contraction coupling[162]. A wide number of hormones and second messengers acting via the cAMP/ PKA pathway can phosphorylate the calcium channel and thus modulate inward

calcium current. At physiological temperature I_{CaL} is larger with more rapid activation and inactivation[161] than at room temperature[155]. ISO, on binding to β-AR, activates G_s-protein adenylylcyclase and via temperature-dependent enzyme cascades culminates in the phosphorylation of dihydropyridine-sensitive calcium channels resulting in increased I_{CaL}[90;147;161]. No changes in I_{CaL} single channel conductance, reversal potential, or the numbers of channels available have been observed in response to β-AR stimulation with ISO[90]. In human atrial myocytes ISO at 1μM almost doubled I_{CaL} current density and resulted in an elevation in the action potential plateau[161]. Relatively little is known regarding the effects of ISO on APD and ERP in human atrial myocytes, and there have been no reports in the literature of the impact of pre-cardiac surgery β-B therapy or heart failure on the effects of ISO on I_{CaL}, APD and ERP with respect to arrhythmogenesis.

1.11 The pro-arrhythmic effects of ISO

Increased adrenergic stimulation alone or in combination with increased rates of electrical stimulation causes an increase in the frequency and amplitude of diastolic calcium sparks[162]. If the SR calcium load is sufficiently enhanced, waves of calcium-induced-calcium-release propagate in cardiac myocytes, activating pacemaker currents and stimulating delayed afterdepolarisations and triggered arrhythmia[162]. Increased cytosolic calcium concentration accelerates I_{CaL} inactivation[161;163] which predisposes to early afterdepolarisations. In addition to effects on calcium handling in human atria, ISO has effects on outward repolarising currents. ISO reversibly increased, in a concentration dependent manner, the delayed rectifier potassium currents I_K[164] and the ultrarapid delayed rectifier potassium current I_{kur}[165]. Furthermore ISO increased the pacemaker current (I_f) amplitude, shifting the voltage of activation towards more positive values in human atrial cells[166] which in combination would increase the probability

of delayed afterdepolarisations and predispose to re-entry in human atrium. The ability of catecholamines to induce afterdepolarisations in human ventricle and animal atrium is well recognised[167]. However despite the many reports of the effects of ISO on isolated currents in human atrium, the pro-arrhythmic effects of isoproterenol to induce afterdepolarisations in isolated human atrial myocytes has not been previously demonstrated.

1.12 Endothelin-1

Endothelin-1 is a relatively short 21 amino-acid peptide first isolated from the cultured supernatant of porcine aortic endothelial cells, unrelated to previously identified vasoconstrictor peptides[168]. The first and most abundant of the three endogenous isoforms to be identified, mature ET-1, is generated by the action of endothelin converting enzyme (ECE) on big ET-1 which has previously been cleaved from intrinsic or circulating preproET-1, a 200 amino acid polypeptide. Endothelin-1 has been demonstrated to have vasoconstrictive effects in several vascular beds in numerous mammalian species including humans[168-171] and has a multitude of other effects; stimulating the release of NO and prostacyclin from vascular endothelial cells[172], stimulating proliferation in smooth muscle cells[173] and myocardial cell hypertrophy[174], in addition to positively inotropic[175-178] and lusitropic[179] effects in human atrial myocytes. Endothelin-1 binds with two distinct endothelin receptor subtypes, $ET\text{-}_A$ which has a greater affinity for ET-1[180] and $ET\text{-}_B$[181] which has almost equal affinity for all three endothelins (ET-1, ET-2 & ET-3). Both endothelin receptor subtypes, $ET\text{-}_A$ and $ET\text{-}_B$, are seven transmembrane domain spanning, G_q-protein-coupled receptors (GPCR) that are widely expressed throughout the normal and diseased human heart[182-184]. In human right atrial specimens the endothelin receptor subtype expression ratio $ET\text{-}_A$: $ET\text{-}_B$ was approximately 2:1[184]. In patients with CHF, levels of ET-1 are elevated[123]. In atria

from failing hearts ECE mRNA was upregulated and ET-1 peptide levels were increased, while ET-$_A$ mRNA was unaltered and ET-$_B$ mRNA was downregulated in failing hearts[185]. The human atrial endothelin system is also altered in AF[131]. The amount of ET-$_A$ and ET-$_B$ receptor proteins were significantly reduced in patients with AF, as was ET-$_B$ mRNA when compared with tissue from patients in sinus rhythm[186]. In human right atrial myocytes ET-1, via ET-$_A$ receptors, inhibits adenylate cyclase via G_i protein and also activates protein kinase C (PKC) via G_q-protein coupled phospholipase C (PLC)[184;187]. This is in contrast to human ventricular myocytes in which ET-$_A$ receptors are not coupled to G_i inhibition of adenylate cyclase[188]. In human right atrial strips, ET-1 in concentration dependent manner from 0.1 nMol to 1 µMol, increased inositol tri-phosphate (IP_3) accumulation but had no effect on cAMP under basal conditions[187].

1.13 The positive inotropic effect of endothelin-1

An endothelin system has been described in human atrial and ventricular myocardium[185;189]. Evidence for an autocrine or paracrine effect of ET-1 in the human heart is growing[56;57;190]. In human cardiac tissue nanoMolar concentrations of ET-1 had a greater positive inotropic effect via ET-$_A$ receptors in right atrial than in ventricular preparations[175;188;191]. The positive inotropic effect of ET-1 is maintained[191] or enhanced[188] in atrial tissue from failing compared to non-failing hearts. The positive inotropic effect of ET-1 results from an ET-$_A$ mediated activation of PKC increasing intracellular calcium concentrations and an increased myofilament sensitivity to calcium via PKC activated increases in Na/H exchanger activity[177;178;188;191-194]. Initially it was speculated that the increased cytosolic calcium concentration required to create the positive inotropic effect of ET-1 was due to an ET-1 induced increase in I_{CaL}. However, subsequently it has been shown that ET-1 can produce a positive inotropic effect in the absence of an increase in

I_{CaL}[195-197]. In rat atrium ET-1 had a powerful positive inotropic effect raising intracellular calcium concentration by 50% in the presence of the I_{CaL} blocker PN 200-110[197]. ET-1, although positively inotropic, was significantly less positively inotropic than ISO[193] in human right atrial tissue. The positive inotropic effect of ET-1 but not ISO was abolished when PKC or Na/ H exchange was inhibited[177]. ET-1 did not activate adenylate cyclase and therefore appears to have a positive inotropic effect independent of cAMP. Thus the positive inotropic effect of ET-1 is mediated differently to that of ISO and potentially independent of I_{CaL} in human atrial tissue.

1.14 The effect of ET-1 on I_{CaL} and action potentials

A direct effect of ET-1 on inward calcium current was suggested in the original paper isolating ET-1 as, in porcine coronary artery, the vasoconstrictor effect of ET-1 was inhibited in the absence of extracellular calcium ions and in the presence of Nicardipine, a selective dihydropyridine calcium channel blocker[168]. Endothelin-1 at 10 nM had no effect on the affinity or density of dihydropyridine binding sites and at 100 nM failed to displace bound radio-labelled calcium channel agonists in rat cardiac membranes suggesting that ET-1 does not interact with the dihydropyridine calcium channel at the level of the membrane receptor[198]. The effect of ET-1 on human atrial I_{CaL} is controversial. In two studies, at room temperature employing the ruptured patch method, ET-1 has been found to increase[199], decrease[199;200] and have no effect[199] on human atrial I_{CaL}. The stimulatory effect of ET-1, where detected, was antagonised by the $ET\text{-}_A$ receptor antagonist BQ-123 and the inhibitory effect of ET-1 antagonised by the $ET\text{-}_B$ receptor antagonist BQ-788[199]. However the $ET\text{-}_A$ receptor antagonist BQ-123 can directly modulate I_{CaL} in animal experiments[201]. It is recognised that temperature affects I_{CaL} and that results from ruptured patch experiments can be difficult to

interpret because of a lack of reversibility of effect and 'rundown' of I_{CaL}. The perforated patch technique permits a more physiological intracellular milieu, prolonging the ability of the cell to respond to neurohormones and avoiding disruption of the normal intracellular calcium buffering mechanisms[202]. Previously apparent controversy in the literature relating to results from animal experiments were resolved by utilisation of the perforated patch technique demonstrating that ET-1 alone under more physiological recording conditions did not modulate basal I_{CaL}[203]. The absence of any effect of ET-1 on basal I_{CaL} is supported by the lack of effect of ET-1 on cAMP under basal conditions in human atrium[187;203].

1.15 The proarrhythmic effect of ET-1

In human cardiac myocytes ET-1 was found to induce arrhythmic contractions in right atrial trabeculae, and those occurred more frequently in tissues excised from patients receiving, when compared with those not receiving, β-B preoperatively[191]. In one small study, ET-1 had no appreciable effect on action potentials in human atrial trabeculae despite the positive inotropic effect remaining intact[176]. However the separation of a vasoconstriction induced ischaemic arrhythmic effect from an intrinsic effect on arrhythmogenesis remains controversial[59;204]. Yorikane[61] first demonstrated that ET-1 causes APD prolongation in the right bundle branch of canine myocardial tissue and that this APD prolongation is followed by early afterdepolarisations, suggesting a direct arrhythmogenic effect of ET-1 on cardiac cells. The direct arrhythmogenic effect of ET-1 has not been studied in human atrial myocytes.

1.16 The interaction of ISO and ET-1 on I_{CaL} and action potentials

In contrast to the differing effects observed of ET-1 alone on basal I_{CaL} in both previous studies in isolated human atrial myocytes, ET-1 (10 nM) had a consistent $ET\text{-}_A$ receptor mediated, pertussis toxin sensitive, anti-adrenergic effect to decrease I_{CaL} following pre-stimulation of I_{CaL} with ISO[199;200]. This effect was irreversible and no data are available to determine whether the effect of ET-1 could be overcome with increasing concentrations of ISO. ET-1, via $ET\text{-}_A$ receptors has been shown to have an anti-adrenergic effect on other currents in human atrial myocytes. The cAMP-activated chloride current ($I_{Cl,\ cAMP}$) which is controversial as it can only be demonstrated under conditions of adrenergic stimulation in some atrial myocyte preparations, was activated with 1μM ISO and subsequently was significantly inhibited by ET-1 (30 nM) by 75%± 6%[205]. This anti-adrenergic effect of ET-1 could be explained by the effect of nanoMolar concentrations of ET-1 to decrease ISO/ forskolin induced increases in cAMP in human right atrial strips. There have been no previous reports examining the interaction of effects of ISO and ET-1 on APD and ERP in human atrial myocytes, and there have been no reports in the literature of the impact of 'pharmacological remodelling' nor CHF on the interaction of effects of ISO and ET-1 on I_{CaL}, APD and ERP.

1.17 The potential mechanisms for ISO and ET-1 to affect arrhythmogenesis in human atrial myocytes

Atrial Fibrillation has been described as multiple re-entrant waves conducted chaotically throughout the atria. Electrical heterogeneity in the atria, whether due to ischaemia, structural heart disease or heart failure is central to the phenomenon of re-entry. Re-entrant waves are formed when electrically coupled tissue has

differing properties of refractoriness or structural discontinuities produce lines of unidirectional conduction block. When a wavefront meets refractory tissue the direction of the impulse changes traversing around these refractory areas of tissue and thus may either be extinguished if the tissue encountered is completely refractory or electrically inert, or propagating daughter wavelets and yet further fibrillatory waves resulting in arrhythmia perpetuation.

Cardiac arrhythmogenesis, however, requires a trigger to initiate an electrical impulse in addition to a substrate of conducting tissue capable of maintaining the arrhythmia. More recently, at the level of the whole heart in vivo, the trigger for AF has been identified in patients as originating in the pulmonary veins[51]. At the cellular level, triggered activity such as early afterdepolarisations (EAD), which are secondary depolarisations occurring prior to full repolarisation of the action potential, or delayed afterdepolarisations (DAD), initiated after myocyte repolarisation is complete, can result in impulse propagation and formation of wavelets capable of being conducted throughout the atria and thus compete with the sinus node for non refractory atrial myocytes.

Prolongation of the APD has been implicated in the generation of EADs[206]. A number of mechanisms have been demonstrated to contribute to this prolongation- a reduction in repolarising K^+ currents resulting in more slowly inactivating I_{Na}[207;208]. Also small calcium transients may result in complete I_{CaL} inactivation increasing the likeliehood of inward I_{CaL} reactivation in the AP and thus EADs[11;209].

Abnormalities in calcium handling have a pivotal role in the generation of DADs. Activation of β-adrenoceptors augments I_{CaL} and increases SR calcium uptake resulting in intracellular calcium overload which in turn increases calcium-dependent-calcium-release from the SR activating arrhythmogenic transient

inward current I_{ti}[210;211]. Atrial myocyte calcium loading is further potentiated in heart failure due to tachycardia and increased 'reversed' sodium-calcium exchange current (I_{NCX}) combined with decreased I_{K1}, thus for any given SR calcium release more I_{NCX} will result in more calcium activated I_{ti} and hence DADs[162;210]. Indeed for a given I_{ti} a greater DAD will result, as a reduced I_{K1} results in a less stable E_m[210]. Thus in heart failure approximately 50% of the normal SR calcium release is required to initiate a DAD of sufficient magnitude to reach threshold and trigger an action potential which may be propagated into an arrhythmia[162].

1.18 Hypothesis

It is my hypothesis that endothelin-1 modulates the effects of isoproterenol on I_{CaL} and consequently action potentials in human atrial myocytes. Such modification may occur at the level of the ligand-receptor complex or via interaction of secondary messenger systems. Any such modification of I_{CaL} by endothelin-1, whether alone or in combination with isoproterenol will have implications for arrhythmogenesis in the human atrium.

2 METHODS

2.1 Ethical Approval

Ethical approval was obtained from the Ethics Committee of Glasgow Royal Infirmary for the use of human atrial tissue for electrophysiology studies from consenting patients undergoing cardiac surgery. This investigation conformed with the principles established in the Declaration of Helsinki [212]. Patients were identified from the cardiac surgery theatre schedule and their consent to participate was obtained.

2.2 Patient Characteristics

Clinical characteristics of all patients from whom samples of right atrial appendage were obtained were recorded in a database (Microsoft Access). Information was obtained from the clinical case sheets relating to age, gender, operative procedure and comorbidity. Data retrieval was complete in more than 97% of instances. In a small number of cases incomplete data was collected due to non availability of complete records. This thesis is based on all available information in order to maximise utility, thus small variabilities in sample size will be presented. Pre-operative drug therapy was assessed from prescription charts. Patients who had been receiving beta-adrenoceptor blocking drugs for less than 30 days pre-operatively were excluded from further analysis. None of the patients had undergone previous cardiac surgery, ablation procedures or were prescribed Amiodarone or Sotalol preoperatively.

Information on cardiac rhythm and rate was assessed using the pre-operative ECG. Only those patients in sinus rhythm with no documented episodes of atrial

fibrillation in the preceding 6 months prior to surgery were included. Postoperatively, case records were later re-examined for evidence of AF up to seven days postoperatively.

2.3 Cell Isolation

The tip of the right atrial appendage was removed at the time of cardiac cannulation for aortopulmonary bypass. The tissue sample was immediately transported to the laboratory, within five minutes of excision, immersed in 50 ml oxygenated warmed modified Tyrode's solution (NaCl 150 mM, KCl 5.4 mM, $MgCl_2$ 1.2 mM, NaHEPES 5 mM, glucose 10 mM, $CaCl_2$ 1 mM; titrated to pH 7.4 with HCl 1M solution). Atrial cells were isolated by enzymatic dissociation and disaggregation using a modified 'chunk' method first described by Escande[213] and subsequently modified by Harding[158] and Workman[79].

Excised tissue was blotted dry and weighed (mass 0.386 $\pm$0.03 g n= 63) then placed on a glass slide, cleaned of visible connective tissue and fat and chopped with scalpels into ~1 mm^3 chunks. These chunks were then transferred to a beaker which was placed into a water bath maintained at 37°C, shaken at 130 strokes per minute and continuously oxygenated in 40 ml nominally Ca-free solution 'B' (NaCl 120 mM, KCl 5.4 mM, $MgSO_4$ 5 mM, pyruvate 5 mM, glucose 20 mM, taurine 20 mM, NaHEPES 10 mM, nitrilotriacetic acid 5 mM and titrated to pH 7.0 with NaOH 1 M solution). The 'chunks' were hand filtered through nylon gauze (200 μm mesh, Barr & Wray, Lanark, UK) and transferred to another beaker containing a fresh sample of 40 ml oxygenated solution ‘B’ at three minute intervals in order to prevent hypoxia and remove any toxic metabolites. After a total of 12 minutes agitation the sample was transferred to another beaker containing 15 ml of

solution 'C' (NaCl 120 mM, KCl 5.4 mM, $MgSO_4$ 5 mM, pyruvate 5 mM, glucose 20 mM, taurine 20 mM, NaHEPES 10 mM, nitrilotriacetic acid 5 mM, $CaCl_2$ 50 μM and titrated to pH 7.0 with NaOH 1M solution) with protease (Type XXIV, Sigma, 4 IU/ml) added and were incubated under identical conditions for a further 45 minutes.

This semi-digested sample was then placed in another 12 ml of modified solution 'C' with collagenase (CLS 1 Type 1, Worthington 330 IU/ml) added in the absence of protease and incubated for consecutive periods of 15, 15 and 20 minutes. Each of these three cell suspensions were filtered through nylon gauze, as before. These three "filtrates" formed three consecutive aliquots which were then centrifuged (Model PK110, A.L.C. International) for two minutes at 40 *g*. The resulting supernatant was aspirated by hand and discarded and the remaining cells were re-suspended for a maximum of 10 minutes in 1 ml of Kraftbrühe (KB) solution[214] (KOH 70 mM, KCl 40nM, L-glutamic acid 50 mM, taurine 20 mM, KH_2PO_4 20 mM, $MgCl_2$ 3 mM, glucose 10 mM, NaHEPES 10 mM, EGTA 0.5 mM and titrated to a pH of 7.2 with 1M KOH solution) in order to wash off any remaining enzymes.

The centrifugation process was then repeated and after removing as much of the KB solution as possible the cells were re-suspended in 1 ml of a low calcium solution (NaCl 130 mM, KCl 4 mM, $CaCl_2$ 0.2 mM, $MgCl_2$ 1 mM, NaHEPES 10 mM, glucose 10 mM and titrated to a pH of 7.4 with 1M NaOH solution). Each of the three aliquots was transferred to a separate petri-dish for storage at room temperature prior to use in experiments. Cells were examined under high power (x40) light microscopy (Nikon TMS microscope) and cells which were isolated, striated, elongated, straight-edged and stable in the perfusion chamber were selected for electrophysiological recordings (Photograph 1 Figure 2.1).

2.4 Electrical Recording Techniques

2.4.1 Patch clamp recording

The extracellular patch clamp method, first described by Neher and Sakmann[215], permits the observation and recording of ionic currents from biological membranes. This technique employs a small highly polished glass pipette containing a predetermined specific 'internal' solution surrounding a silver filament electrode attached to an amplifier and recording equipment. The pipette was advanced into the external solution containing the cell to be examined under positive pressure to prevent contamination. The tip of the pipette was pressed gently against the cell membrane in order to form an electrical seal. The electrical resistance of the pipette-membrane seal obtained by this technique was increased from 50 MΩ[215] to approximately 100 GΩ, using micro-pipettes of smaller tip diameter and the application of negative pressure to the cell membrane as contact was achieved as advised by Hamill et al[216]. The advantage of a pipette-membrane seal of greater resistance, termed a giga-seal, is that background electrical noise or interference is reduced, less current 'leaks' around the pipette into the perfusion chamber reducing inaccuracy of measurements and the mechanical stability is improved.

However, this 'standard' ruptured patch whole cell recording technique, also called the continuous single electrode voltage clamp (cSEVC), has been shown to suffer two major drawbacks when investigating I_{CaL}. The cell membrane is physically disrupted, or intentionally ruptured, using negative pressure permitting washout and disruption of intracellular calcium buffering mechanisms resulting in rapid rundown of I_{CaL} magnitude, alteration of the I: R relationship and desensitisation to adrenergic receptor activated second messengers[202;217-219]. This problem was overcome by adding the polyene antibiotic, nystatin, to the internal solution (see

below) in the pipette. Nystatin containing pipette solutions have previously been demonstrated to form voltage independent, highly selective monovalent cationic pores of 0.4 nM diameter in cell membranes without compromising the cell's integrity[202], increasing the signal to noise ratio, preventing I_{CaL} rundown[220;221] and maintaining sensitivity to isoproterenol for up to 90 minutes recording[221]. The combination of a high seal resistance around the pipette with the small 0.4 nM pores within the pipette permit only monovalent ions and substances up to 200 D molecular weight to dialyse with the internal pipette solution, establishing stable electrical access with preservation of the normal intracellular cytoplasm. Nystatin itself does not enter the cell nor does it cross the glass membrane seal, however it is not without its disadvantages. Nystatin solution loses its pore-forming ability within 4 hours at room temperature requiring fresh stock solutions during an experimental day. This becomes obvious by the inability to obtain satisfactory series resistance within approximately 20 minutes. Successful pore formation precludes any manipulation of the intracellular constituents of the cell under investigation via the patch pipette, limiting opportunities for experimentation. In addition the formation of junction potentials and subsequent intracellular hypertonicity during long experimental protocols can occur[202]. This can be minimised by ionic substitution in the internal pipette solution, use of electrodes of low resistance and effective delivery of sufficient nystatin to the tip of the pipette to ensure excellent seal and low series resistance[202].

Thus the nystatin-perforated whole cell patch clamp technique[202] was employed both to limit I_{CaL} rundown[221] and also preserve the internal milieu of the myocyte[202] during experimental protocols. In order to limit vibration artefact cells were permitted to sediment in the perfusion bath (RC-24E fast exchange perfusion chamber, Warner instruments) (Figure 2.1) positioned on an air suspension table

(Wentworth laboratories) (Figure 2.2) coupled with an Axopatch-1D amplifier (Axon instruments) (Figure 2.3).

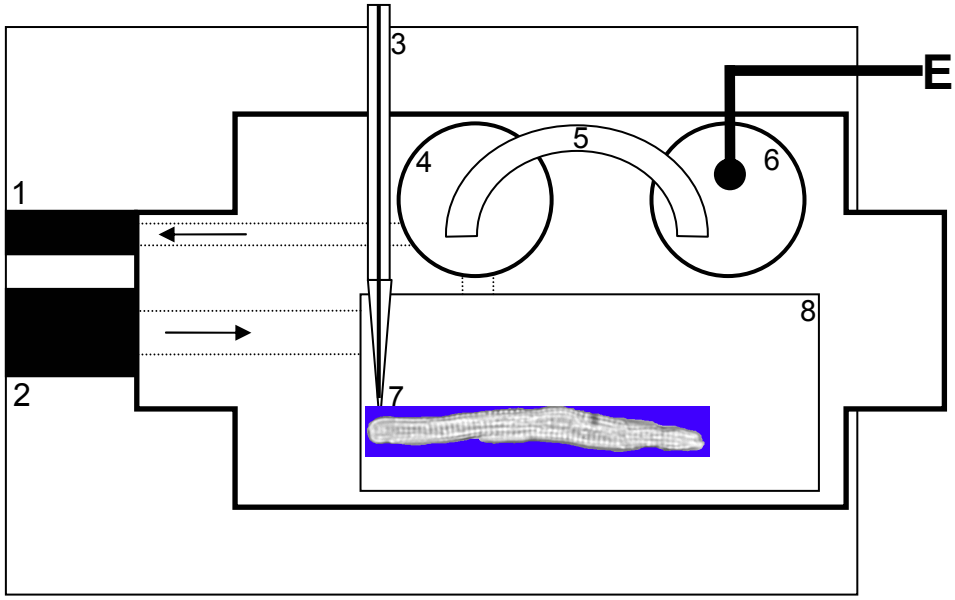
2.4.2 Micropipette electrodes

Fine borosilicate glass pipette microelectrodes (Clark Electromedical) were constructed by hand during cell sedimentation using a vertical micropipette puller (Narishige PP-83) using a two stage thinning process and then heat polished to resistances (R_p) of 2-5 MΩ (mean R_p= 2.7 ±0.1 MΩ n= 148). First the capillary was loaded onto the micropipette puller and thinned at high power increasing capillary length by approximately 8 to 10 mm. Then the thinned capillary was re-centred at the heating element and low power heating used until the capillary split into two identical pipettes. Each of these pipettes was then loaded in turn onto the heat polisher under direct visualization via light microscopy. The tip of the pipette was manoeuvred close to the glowing filament for a few seconds causing a subtle change in shape and a darkening of the silhouette. Pipettes with resistances in the range of 2- 5 MΩ have an internal opening diameter of ≤1 μm[216]. The optimal shape of the micropipette is depicted in Figure 2.4A. The R_p associated most frequently with successful electrical access to the atrial myocyte was found to be 2 MΩ as depicted in Figure 2.4B.

The electrode was formed from a silver wire which was polished to ensure a clean surface prior to being coated by silver chloride (Ag/AgCl) by anodising in a solution of sodium hypochlorite (NaOCl) prior to loading on the head stage (Figure 2.3).

2.4.3 Solutions- Internal

Micropipettes were back-filled with a caesium based 'internal' solution (Cs-methanesulphonic acid 100 mM, NaCl 5 mM, CsCl 30 mM, $MgCl_2$ 1 mM, NaHEPES 5 mM, pH 7.4 with 1 M CsOH) in order to block K^+ currents during voltage clamp experiments recording I_{CaL}. For action potential recordings with the amplifier in current clamp mode micropipettes were back-filled with a potassium based internal solution (K methanesulphonic acid 100 mM, NaCl 5 mM, KCl 30 mM, $MgCl_2$ 1 mM, NaHEPES 5 mM, pH 7.4 with 1 M CsOH). Nystatin (Sigma 184 μM) was dissolved in DMSO (Sigma 120 μL) and added to fresh internal pipette solution at hourly intervals in order to maintain the selective pore forming ability of the Nystatin solution[202].

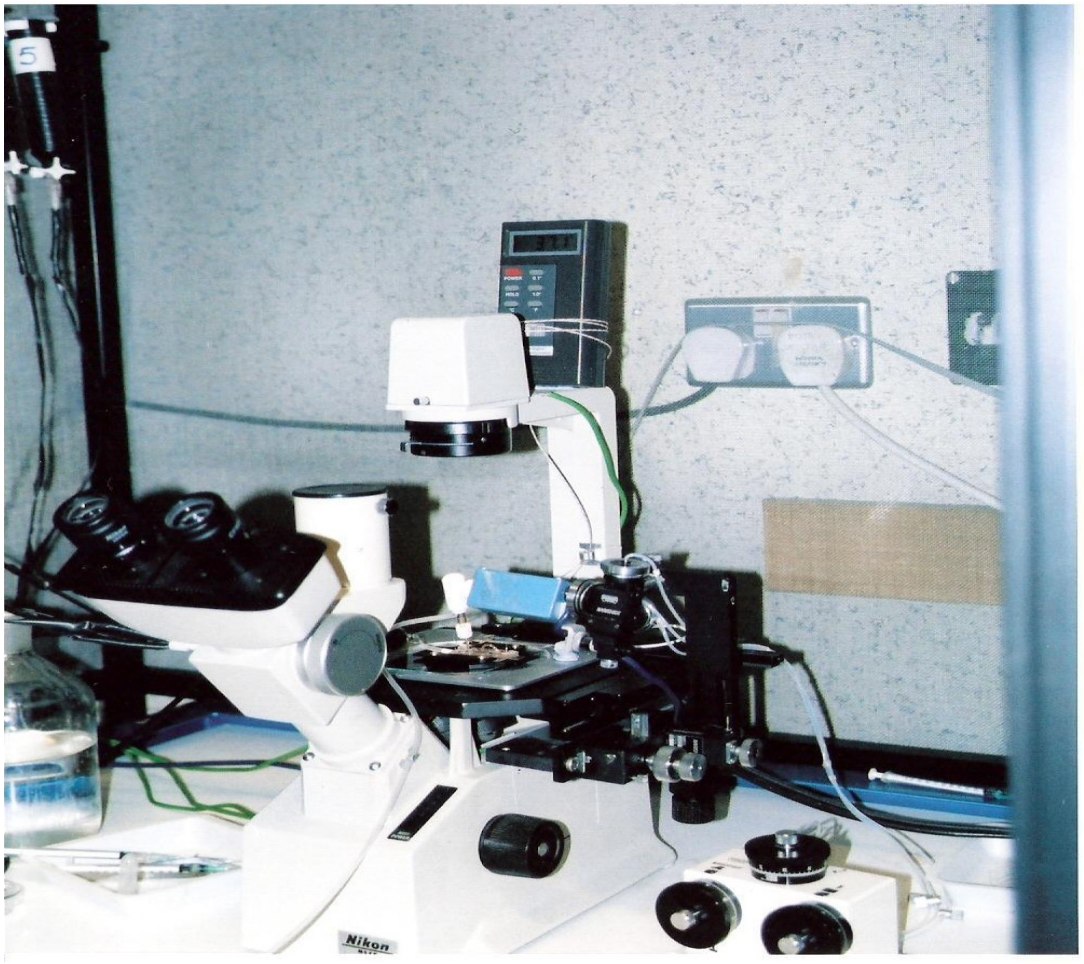


1 Vacuum pump evacuator
2 Superfusate inlet from in-line heater
3 Micropipette Ag/AgCl electrode
4 Evacuation reservoir
5 Salt bridge
6 Earth reservoir (3M KOH)
7 Myocyte forming patch
8 Myocyte perfusion chamber

Figure 2.1 Schemata of the perfusion chamber apparatus with a photograph of human atrial myocyte (not to scale)

2.4.4 Solutions- External

Cells were superfused in the sedimentation chamber with a physiological 'external' salt solution (NaCl 130 mM, KCl 4 mM, $CaCl_2$ 2 mM, $MgCl_2$ 1 mM, NaHEPES 10 mM, glucose 10 mM, titrated to pH 7.4 with a 1 M NaOH) at 1.5 ml/ min maintained at 37ºC via an in-line heater (SH27-B, Warner instruments) as determined by a digital thermometer (Model 410, Rapid electronics). Reagents were stored in lightproof sealed containers in a commercial refridgerator as per the manufacturers' recommendations. On the morning of the experiment pre-specified amounts of reagent were weighed and dissolved in de-ionised water which had been micro-pipetted to the required volume. Reagent solutions were then diluted to the desired concentration using de-ionised water and stored in lightproof sealed



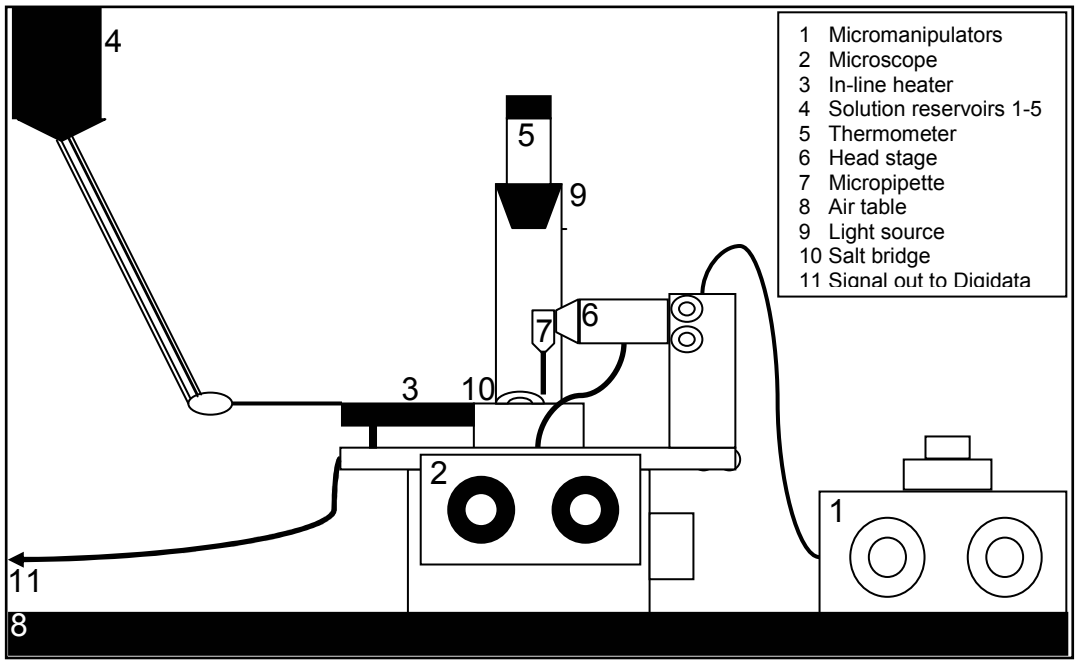
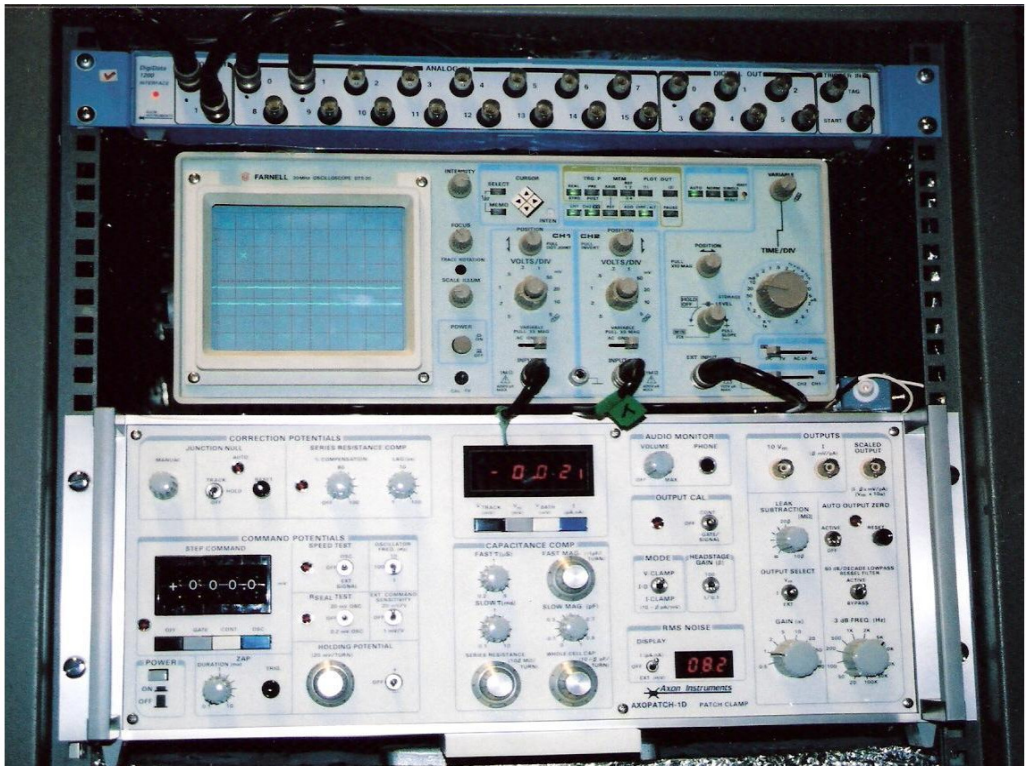


Figure 2.2. Schemata representing electrophysiology recording rig with photographic image.



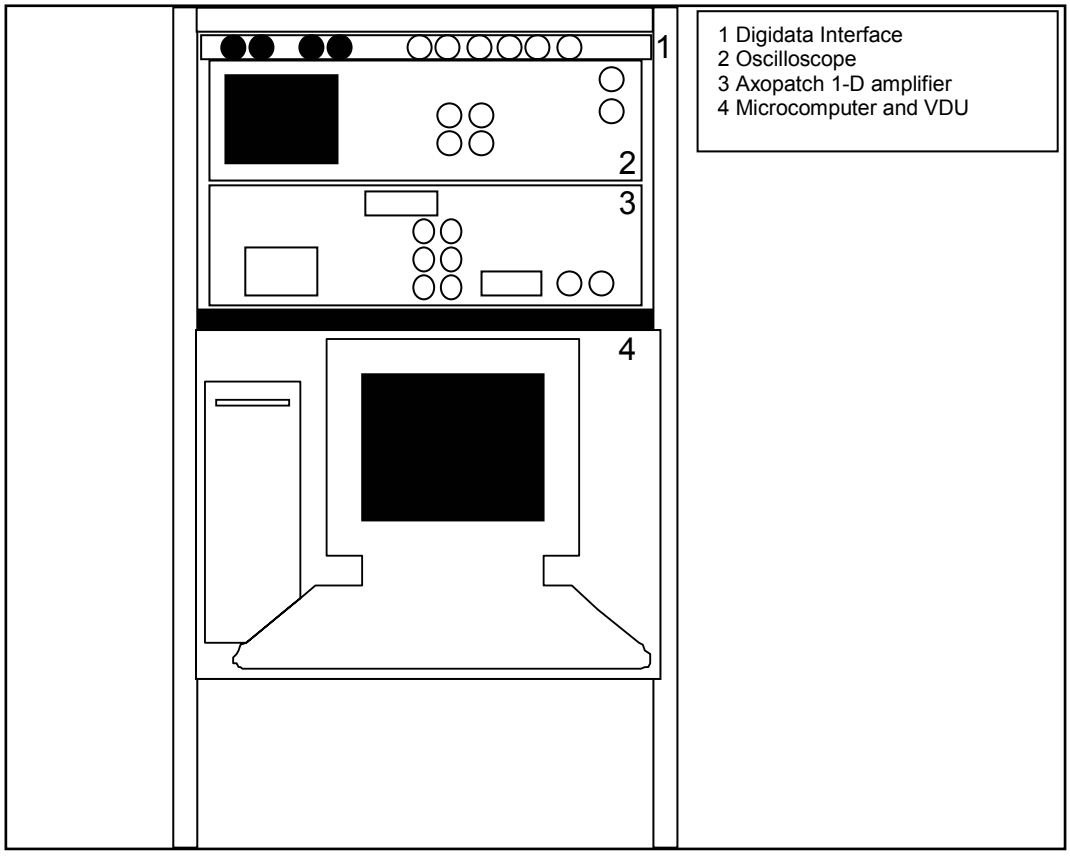


Figure 2.3 Schemata representing digital recording equipment.

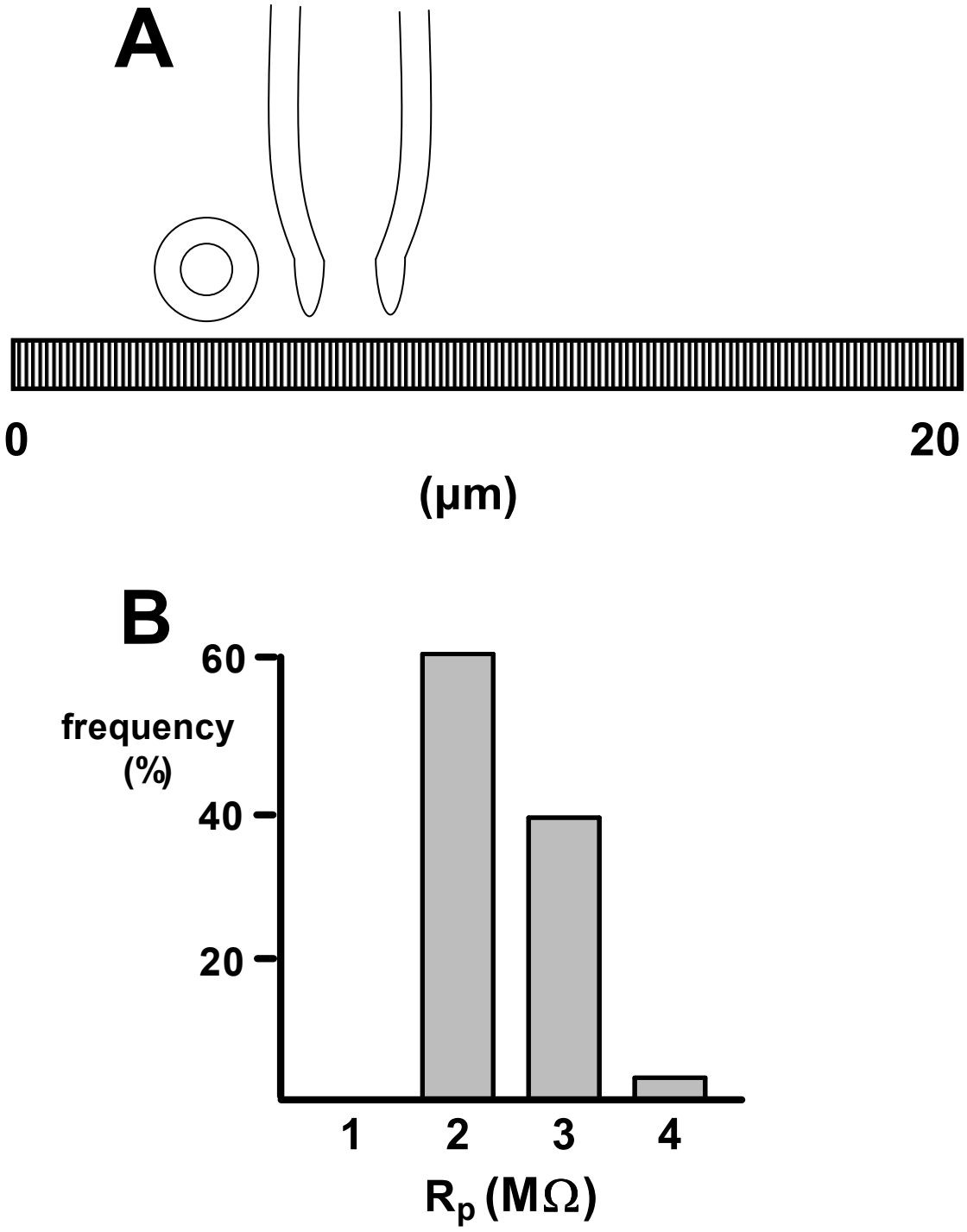


Figure 2.4 Schemata of Micropipette manufacture and frequency distribution of pipette resistance (R_p)
A Schemata to scale of longitudinal and end on appearances of optimal micropipette
B Frequency distribution of pipette resistance (R_p) associated with successful electrical access to the cell (Mean R_p of successful seal formation 2.7 ±0.1 MΩ n= 148 pipettes).

containers attached to the perfusion chamber for the duration of the experiment at room temperature. During superfusion the in-line heater ensured that the ambient temperature within the perfusion chamber was maintained at 37^{o}C (as above).

2.4.5 Liquid Junction Potentials

Using this configuration of solutions with the earth electrode maintained in a 3M KCl bath connected via an agar salt bridge to the perfusion chamber a liquid junction potential (LJP) of +5 mV (±0.1 mV n=5) was measured (bath relative to pipette) using the method described by Neher[222] as follows. The patch clamp rig was prepared as above with both the micropipette and perfusion chamber filled with internal solution. The Axopatch amplifier was switched to current-clamp mode, held at 0 pA, and the offset voltage nulled manually. The perfusion chamber was then evacuated and refilled with external solution. The voltage reading was permitted to stabilise and this solution specific LJP was recorded in the lab book. This recorded liquid junction potential was compared with other work performed in this laboratory[79;223] and found to correlate well. This method of calculating the LJP permits a priori compensation, prior to seal formation, of the LJP by WinWCP software by inserting a junction potential correction of equal size but opposite polarity, however it mandates that the 3M KCl solution in which the salt bridge is bathed is refilled at frequent intervals during the experimental process.

2.4.6 Myocyte selection

The perfusion chamber was thoroughly examined with the microscope, initially on low power, to determine the most suitable myocyte for patch clamp recordings. Myocytes were selected as suitable for recordings if positioned in the perfusion chamber within reach of the pipette and if they remained geographically stable.

Cells which remained isolated, elongated, and striated under high power microscopy during superfusion with control solution containing 2 mM calcium (a 10 fold increase from the low calcium storage solution) were selected. Myocytes were approached obliquely under light microscopy maintaining slight positive pressure in the pipette in order to avoid surface contaminants increasing the resistance of the microelectrode and consequently inhibiting seal formation. The pipette was advanced to within micrometres of the sarcolemma using a micromanipulator (Narishige NW-3) under gentle positive pressure per orum via a small rubber tube with two O rings attached to the micropipette mounting and then when optimally positioned vis a vis the myocyte gentle negative pressure was applied to attach the tip of the pipette to the cell membrane until a gigaohm seal was obtained. The giga-seal was not lost on any occasion when the negative pressure on the pipette was released prior to electrical compensation.

2.4.7 Series Resistance (R_s) and Capacitance (C) Compensation

When the membrane potential was manipulated, there was a significant current transient required to charge the membrane capacitance during which it is impossible to record meaningful data. The settings of the whole-cell capacitance controls to eliminate this capacitative transient are unique to the cell being clamped. The values of the cell membrane capacitance and the access resistance can be directly read from the Axopatch amplifier controls. The absolute value of the membrane capacitance was displayed on the whole-cell capacitance dial after the whole-cell current transient has been eliminated. This value may be used to estimate the surface area of the cell assuming that the membrane capacitance per unit area is 1 $\mu F/cm^2$. Capacitance compensation was used to reduce the effective value of any remaining capacitance error. The Axopatch amplifier injected ‘extra’ current into a capacitor connected to the input. At the ideal setting of the amplifier

gain, the current injected is exactly equal to the current that passes through the stray capacitance to ground hence eliminating error. However, this compensation must be implemented cautiously for if the amplifier gain is increased beyond the ideal setting, the current injected back will cause the input signal to overshoot resulting in circuit oscillation and mycocyte death.

When electrical access to the cell was gained the internal pipette solution formed an electrical continuity with the interior of the myocyte. However, this continuity was imperfect and there remained a small electrical resistance across the pipette of the order 1-10 MΩ. This resistance is termed the series resistance R_s and is electronically compensated in a positive feedback loop by increasing the command potential proportional to the potential drop across the pipette. The R_s must be minimised as it introduces both a voltage error and also lowers the temporal resolution of the voltage clamp. In the steady state, the amplifier will clamp the potential across the pipette while one is aiming to clamp the potential across the cell membrane resulting in an error which can be minimised by maintaining as small a R_s as possible. During depolarisation, rapid step changes (see below) in potential initiated by command from the amplifier will produce changes in cellular V_m with a time lag potentially of milliseconds duration. In order to overcome this problem the patch clamp amplifier continuously computes the potential drop across the pipette and injects a correction signal to the command, effectively reducing the error by a factor of (1- correction factor) thus R_s of 80% correction results in an 80% reduction in voltage error and an 80% reduction in time lag. Caution must be exercised, however, as compensation was increased the stability of the electrical circuit decreased and oscillations occurred resulting in myocyte death above 80% R_s compensation.

The Series Resistance (R_s) (mean R_s= 7.2± 0.2 MΩ n= 255 cells from 67 patients) and Capacitance (C) (mean C= 82.9± 0.8 pF n= 255 cells from 67 patients) of these selected myocytes were measured and electronically compensated (mean R_s% compensation = 68.2± 0.2% n= 161) following the formation of the perforated patch and prior to recording.

Cells were rejected if, after stabilisation, the R_s was >20 MΩ due to potential voltage errors in recording accuracy within the Axopatch 1D amplifier. The software programme utilised, WinWCP (J. Dempster, Strathclyde University) on a microcomputer (Gateway microcomputers with Pentium III microprocessor) permitted simultaneous stimulation and recording. Current and voltage data were low passed filtered at 5 kHz and digitised at a rate of 15 kHz (Digidata 1200 A-D converter, Axon instruments) prior to storage on compact discs.

2.4.8 Resting Membrane Potential (V_m)

Following R_s and C compensation the cell was permitted approximately 60 seconds quiescence following which the resting membrane potential under control conditions (V_m) was recorded in current clamp mode. This was performed by nulling the voltage clamp and recording the potential difference across the myocyte membrane prior to recording I_{CaL} or action potentials. The mean V_m for all cells was -19± 4 mV (n= 81 cells from 23 patients).

2.5 Experimental Protocols

2.5.1 Voltage Clamp: I_{CaL} current: voltage relationship

During voltage clamp experiments the membrane voltage of the myocyte under investigation is held constant and the trans-membrane current required to maintain that voltage across the cell membrane is measured. This artificial process, if performed carefully, eliminates the capacitive current bar the immediate voltage step and permits control of activation and inactivation of voltage dependent I_{CaL}. The resultant recorded current is directly proportional to the membrane conductance of I_{CaL}.

Once a giga-seal was obtained and electrically stable access to the cell achieved the cells were voltage clamped at a holding potential (HP) of -40 mV. This was chosen in order to avoid contamination of I_{CaL} by rapid and large inward Na^+ currents which might be present at more negative potentials. The current-voltage (IV) relationship of I_{CaL} was determined using repeated stepwise incremental +10 mV depolarising pulse steps of 350 ms duration every 3 seconds from the holding potential of -40 mV up to a maximum of +60 mV at physiological temperature (37°C) (See Figure 2.5). This physiological temperature was chosen as it has previously been shown that I_{CaL} has temperature dependent time course of activation and inactivation[161]. Previous work examining I_{CaL} has demonstrated that a depolarising stimulus frequency of 0.33 Hz permits full recovery of I_{CaL} prior to the next depolarising pulse[94].

2.5.2 Voltage Clamp: peak I_{CaL} time course effects

Experiments examining the time course of the effects of both ISO and ET-1 were performed using a -40mV Holding Potential with repeated identical voltage steps from a HP of -40 mV to +10 mV of 350 ms duration at a fixed frequency of 0.2 Hz while the perfusion chamber was maintained at 37°C (Figure 2.6). This step potential was chosen as the previously obtained I_{CaL} IV relationship peaked at +10 mV which is consistent with other reports in the literature using similar experimental conditions [79;161].

2.5.3 Current Clamp: action potential recordings

During a current-clamp experiment a constant or pre-specified time-varying current is applied to the myocyte and the resultant change in membrane potential is measured. Technically more challenging than voltage clamping in the laboratory, current clamping recreates the response of a myocyte to electrical stimulation or depolarisation in vivo, that is, the generation of action potentials.

Myocytes in the resting state were current clamped to -80± 5 mV and subsequently the holding current was maintained <150 pA thereafter. Action potentials were stimulated at 75 beats per minute (bpm) using 5 ms current pulses of 120% threshold strength. The stimulus threshold was initially determined by repetitively stimulating the cell with a train of three 5 ms current pulses, the first and second stimuli being of equal supra-threshold amplitude and the third pulse of lesser amplitude increasing in 50 pA increments from 0 pA until a regenerative action potential was stimulated.

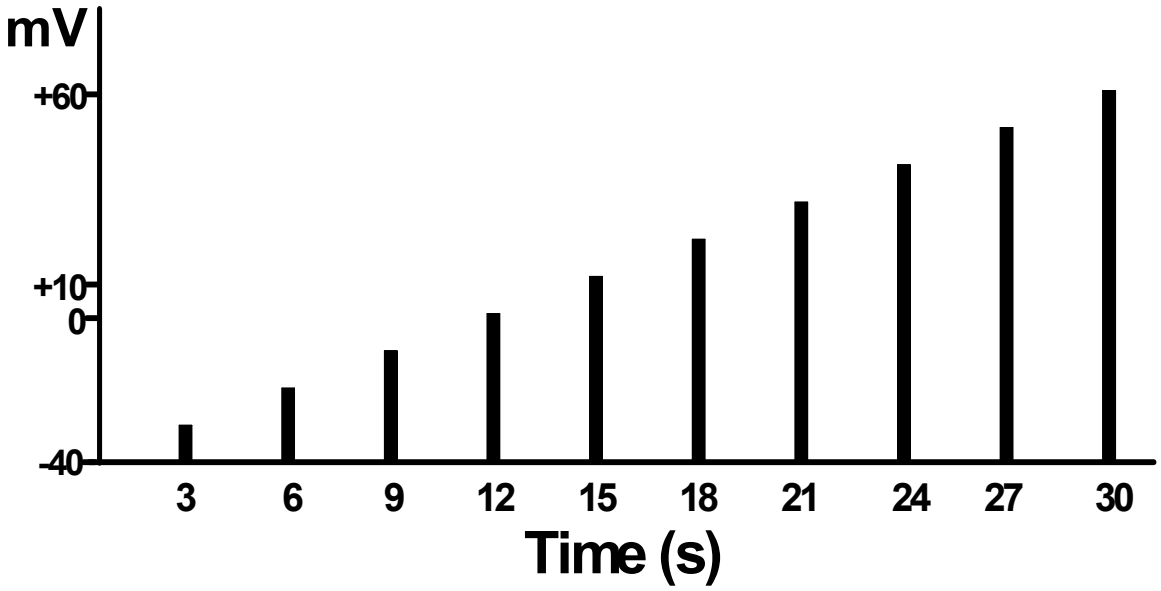


Figure 2.5 Diagram of the protocol for determining the current voltage relationship of I_{CaL}.

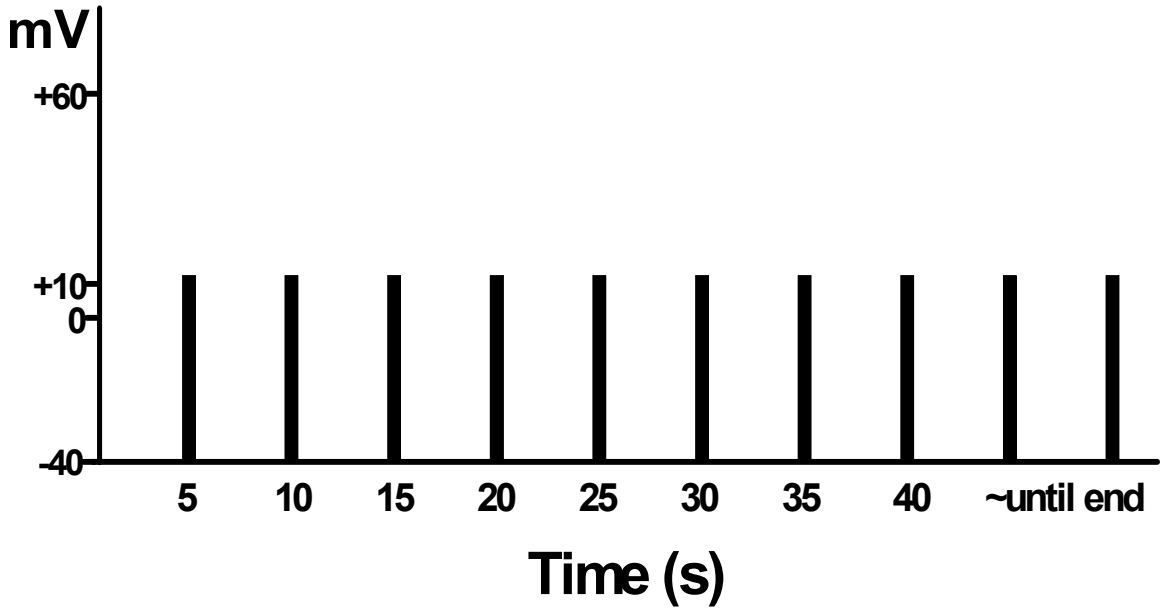


Figure 2.6 Diagram of the protocol for determining the time course of effects on peak I_{CaL}.

This threshold was then increased by a factor of 1.2 and this 'supra-threshold' stimulus was maintained constant throughout the experiment in that cell (See Figure 2.7). The entire train of stimulated action potentials was visible on the oscilloscope, however, in order to maximize resolution, only the final two S_1 action potentials and the S_2 action potential were recorded. The APD was calculated as the interval between the action potential upstroke and repolarisation to the level of

50% (APD_{50}) and 90% (APD_{90}) of the upstroke amplitude (See Chapter 5 Methods section).

2.5.4 Current Clamp: Measurement of the effective refractory period (ERP)

The ERP of each cell was determined using a standard S_1-S_2 protocol consisting of an 8 pulse conditioning train of 5 ms pulses at 75 bpm (S_1) followed by one pulse of equal magnitude and duration which was delivered at 10 ms progressively shorter intervals (S_2) (See Figure 2.7). The cellular ERP was defined as the shortest S_1-S_2 interval which elicited a regenerative action potential of amplitude greater than 80% of the preceding S_1 action potential.

2.5.5 Current Clamp: Spontaneous depolarisations

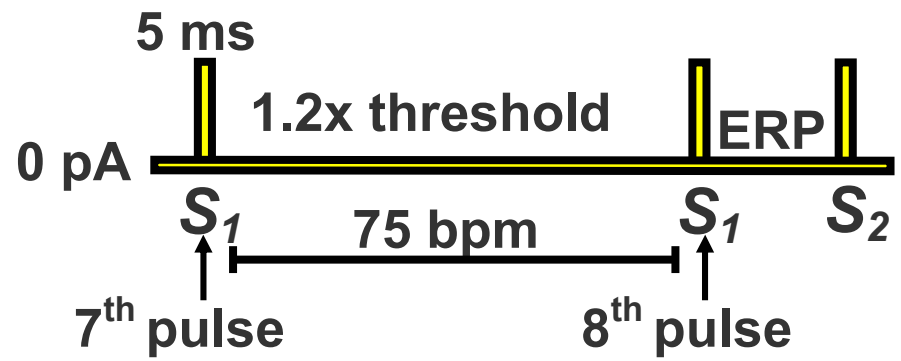
During ERP experiments it was noted on the oscilloscope that following superfusion with 0.05 μM ISO a number of cells became electrically unstable. On inspection of this phenomenon it appeared that during the train of S_1 stimuli the appearance of 'spontaneous depolarisations' preceded electrical instability. The ERP protocol was modified to a repetitive protocol ('REP') in which the final S_2 stimulus was replaced with another S_1 stimulus and the entire train was recorded (See Figure 2.7).

2.6 Data Analysis and Statistics

WinWCP raw data records were analysed visually and an absolute value of I_{CaL} for every record was obtained by subtraction of the steady state current at the end of the depolarising step from the peak inward current. The magnitude of peak I_{CaL} absolute current (pA) was corrected for cell capacitance (pF) and the consequent

I_{CaL} current density (pA/pF) was used to control for variation in cell size. This current density value measured in WinWCP was recorded and stored in Microsoft Excel and transferred into the data records of Graphpad Prism software. Mean data pertaining to individual myocyte recordings in WinWCP traces were obtained using this Prizm software and related mean data graphs constructed.

Myocytes, wherever possible, acted as their own control. Sample size was primarily determined by number of myocytes, however the number of human subjects required to provide samples of right atrial appendage from which these cells were isolated was also recorded. Data relating to I_{CaL} were expressed as mean± standard error of the mean (s.e.m.) with n_c= number of cells studied and n_p= number of patients from whom these cells were obtained. The mean values were compared using a two-tailed paired or unpaired Student's *t*-test as appropriate. A Chi-squared (χ^2) test was used to assess the level of significance of differences in the incidence of events between groups. Statistical significance was predetermined at a level of $P< 0.05$ throughout.



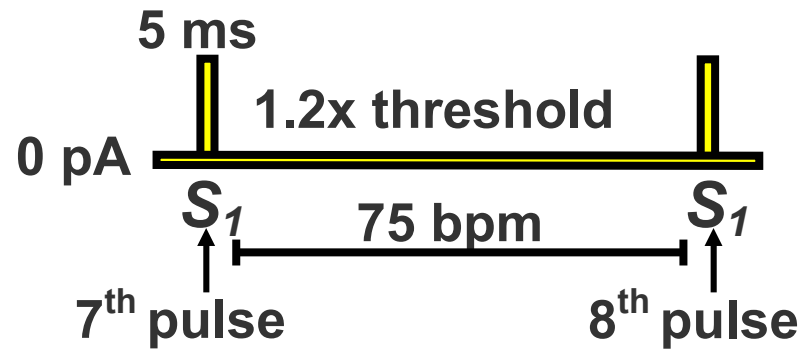


Figure 2.7 Diagrammatical representations of the current clamp protocols.
A Schemata of the protocol used to record Action Potentials and the Effective Refractory Period.
B Schemata of the protocol used to record Action Potentials and Spontaneous Depolarisations.

3 RESULTS (i)

L-type calcium current recordings

3.1 Introduction and aims

L-type calcium current (I_{CaL}) is the large, voltage and time dependant, long-lasting inward current, by convention designated with a negative polarity, which enters atrial myocytes during the plateau phase of the action potential. L-type calcium channels are heterotrimeric proteins which span the plasmalemma and are activated by the voltage changes occurring during the initial depolarisation phase of the action potential under physiological conditions. In human atrial myocytes the inward calcium current contributes to the plateau phase of the action potential and this calcium entry triggers calcium-induced-calcium-release from the sarcoplasmic reticulum resulting in myofilament contraction[158;162]. Numerous roles have been attributed to I_{CaL} in the human cardiovascular system and this current is of particular interest because of its role in excitation-contraction coupling and arrhythmogenesis[224;40;162;163].

Although extensively studied in human ventricular myocardium fewer studies have sought to characterise this current in human atrium and the majority of those have investigated current characteristics at room temperature. Modification of the function or density of human atrial I_{CaL} has been associated with chronic drug therapy[94;114;155] and disease states[113;115;158] and these altered electrophysiological characteristics have been postulated to be both protective against and contributing factors in altered contractile function and arrhythmogenesis[162].

Human atrial I_{CaL} has been described by a small number of groups who have used the ruptured patch clamp technique either at room[114;155;163;225] or physiological temperature[79;144;161;223], and although broadly similar results have been reported important differences in current characteristics have emerged[161].

The effects of adrenoceptor agonism by ISO in human right atrium both in vivo[226] and in vitro[146;227-233] have long been recognised as a potential mechanism for investigation of human ion current characteristics[234], indeed many early reports describe augmenting inward calcium current in order to reproducibly identify calcium currents[155;235]. The increase in I_{CaL} inward current flow due to β-AR stimulation is accepted to be due to an increase in the probability of a calcium channel being open as a result in an increase in the fraction of calcium channels available to open during depolarisation in the absence of increased single channel conductance, altered reversal potential or the total number of channels per unit membrane[236-239]. In contrast, the stimulatory effect of catecholamines on I_{CaL} at physiological temperature with the perforated patch technique[92;114;155;161;163;199;224;240] has not previously been studied and differences in temperature and recording techniques have previously been shown to alter ion current characteristics considerably[161].

Endothelin, a 21 amino-acid peptide, was first discovered in 1988 as a potent vasoconstrictor in the supernatant of cultured porcine aortic endothelial cells[168]. Acting in concert with other neurohormones, such as Angiotensin II (A-II) and adrenergic stimulation, ET-1 released either in an autocrine or paracrine manner, has direct and indirect effects on inotropy[241-243], chronotropy[241-243], myocyte hypertrophy[244] and I_{CaL}[245-249].

Initial experiments with intra-aortic endothelin infusions resulted in a concentration dependent reduction in coronary perfusion and thereafter induced a concentration dependent negative chronotropic and inotropic effect on Langendorff mounted rat hearts. The threshold effect of ET-1 was in the nanoMolar range but even with nanoMolar concentrations, experimental models have struggled to divorce the electrophysiological effects of ischaemia resulting from ET-1 induced coronary vasoconstriction from any direct intrinsic inotropic effect[250].

It has long been noted that plasma endothelin levels are elevated in CHF[123;125;128;132;251;252] and correlate with poor functional status and prognosis[123;125;128;129;251;253;254]. Circulating levels of ET-1 in plasma from patients with CHF can be as high as 10 pg/ml[253;255] and have been demonstrated to be similar in right and left atria[256]. In human patients with CHF circulating plasma ET-1 concentrations correlate with right atrial pressure and decrease in response to BB therapy[132] occasionally returning to normal levels if treatment renders the patient asymptomatic[123;125;252;254;257]. Whether elevated circulating levels in plasma or local concentrations of ET-1 are primarily important in determining the physiological effects of ET-1 remains open to debate. Intuitively, as ET-1 is thought to act in an autocrine or paracrine matter, local concentrations are deterministic. The time course of neurohormonal activation in patients with CHF is not known.

Endothelin-1 has been shown in animal models to be both pro and anti-arrhythmic depending on the physiological setting and animal model under investigation. Initial reports described stimulatory[196;248;258;259], inhibitory[195;196;248;260-263] or no effect[203;247;249;264] of ET-1 on I_{CaL}, with an EC_{50} between 1 and 10 nM, mediated via $ET\text{-}_A$ receptors in animal models[195;196;203;248;249;258-264]. In addition to the possible effects on calcium current, activation of the Sodium-Hydrogen exchange current

(NHE)[265] and Sodium Calcium Exchange current (NCX)[266-268] via PKC, inhibition of K^+ outward currents[62;269-272] and I_{Cl} via inhibition of PKA/ adenylate cyclase[273] have been described.

Despite promising results from trials of long term ET-$_A$ receptor blockade in animals[274;275], results of trials in human patients with CHF have been disappointing[276] indeed two larger studies identified an unexpected increase in atrial[277] and ventricular[278] arrhythmia prior to early termination.

CHF is associated with systemic circulating elevated ET-1 levels, although the local levels in the human atria during CHF are not known. ET-1 is reported to have potential direct and indirect electrophysiological actions but further study is required. CHF is also associated with progressive cellular and electrophysiological remodelling that predisposes to AF. We sought to identify the acute electrophysiological effects of ET-1 on human atrial myocyte I_{CaL} and, later, action potentials to determine whether this could in part explain CHF induced electrophysiological remodelling.

The aims of these experiments were as follows:

- To determine the range of concentrations of ISO which would reproducibly augment I_{CaL} under the recording conditions being used and define the concentration: response relationship.
- To select a concentration of ISO approximate to the EC_{50} which would reliably 'pre-stimulate' peak I_{CaL} resulting in a sufficiently stable peak current to permit interpretation of any subsequent effect(s) of ET-1 or its analogues on peak I_{CaL}.

- To assess the impact of the nystatin-perforated as compared to the nystatin-ruptured patch technique of electrical access on the stimulatory effect of ISO on I_{CaL}.
- To investigate whether patient senescence, chronic preoperative drug therapy or pre-existent comorbidity were associated with an altered adrenergic stimulatory effect of ISO on I_{CaL}.
- To investigate the effect(s), if any, of ET-1 on I_{CaL} and to determine the ET-1 receptor(s) subtypes involved in mediating any ET-1 effect on I_{CaL}.
- To investigate the effect(s) on I_{CaL} of ET-1 and ISO in combination.

3.2 Methods

The nystatin-perforated whole cell patch clamp technique was used to record I_{CaL} as described in detail in Methods section 2.4. Data were obtained with the patch clamp amplifier in voltage clamp mode using a Caesium based 'internal' solution in order to block K^+ currents.

Following examination of the I_{CaL} traces obtained in the early experiments it was apparent that stable recordings obtained with a sufficiently good seal were associated with a greater inward current than 'leak' tail current. The ratio of leak tail current to peak inward current was assessed first subjectively and then calculated objectively and if this 'leak to peak' ratio (LtP ratio) was greater than 1 the cell was excluded as shown in Figure 3.1A.

Figure 3.1B illustrates an acceptable recording of I_{CaL} under control conditions. Following a voltage step of 350 msec duration at 0.2 Hz from the holding potential -40 mV to +10 mV, while the cell and the superfusate are maintained at 37°C, a rapid and reproducible increase in inward calcium current occurred which reached

a maximum in less than 5 ms and which appeared to decay in a bi-exponential fashion. Subtraction of the steady state current at the end of the depolarising step from the peak inward current permitted estimation of the absolute magnitude of I_{CaL} for any given record. Recordings from cells were excluded from further analysis if this LtP ratio was ≤ 1, such as that depicted in Figure 3.1A.

In early experiments electrical artefact and rapid I_{CaL} rundown in addition to 'leak to peak' ratio suggested a time cut off of March 2002, approximately two months (eight experiments) into the project. This date was selected as, in retrospect, stable recordings associated with a 'leak to peak ratio' of less than 1 were in the minority until that date and is taken to reflect the author's 'learning curve'.

Cells were not immediately excluded, pending subsequent analyses, if electrical access to the cell was formed abruptly, i.e. ruptured, as opposed to perforated, patch clamping (Figure 3.2). Cells in which the R_s dropped so rapidly as to preclude simultaneous R_s compensation (Figure 3.2A) were designated as Nystatin Ruptured (NR) cells and comprised 29% of the total myocyte recordings. A comparison between the time courses of ruptured and perforated seals can be seen in Figure 3.2. The mean time course of the drop in series resistance (R_s) during the formation of perforations in all cells is depicted in Figure 3.3.

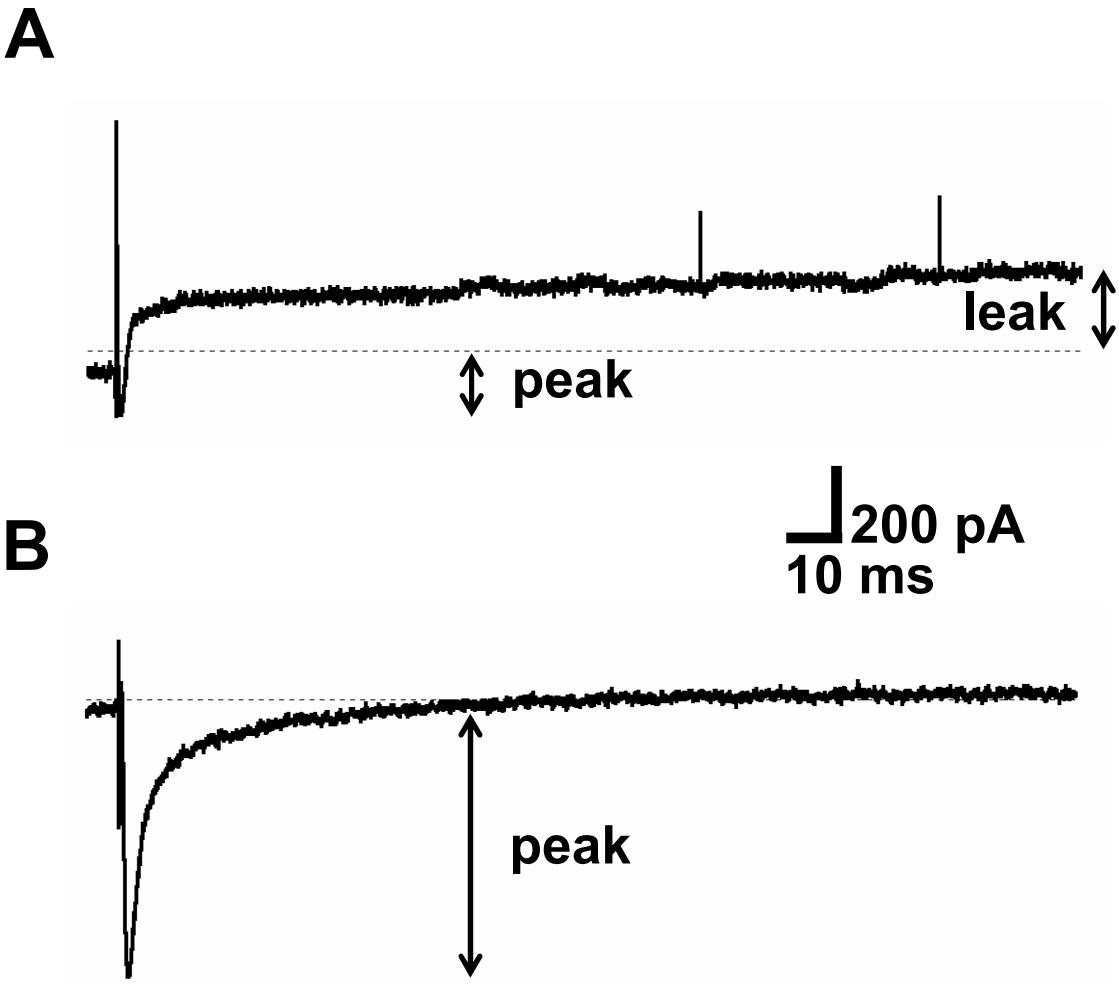


Figure 3.1 Variability of I_{CaL} recordings: demonstration of leak to peak exclusion criterion.
A peak I_{CaL} current trace from human atrial myocyte depolarised for 350 msec from a holding potential of -40 mV to +10 mV at physiological temperature using the nystatin perforated patch clamp method demonstrating a peak I_{CaL} which when corrected for leak current is less than or equal to 1.
B I_{CaL} current trace obtained using identical methodology exhibiting leak to peak ratio greater than 1 and hence excluded from further analysis.

$$\text{Leak to Peak ratio} = \frac{\text{peak current (pA)}}{\text{leak current (pA)}}$$

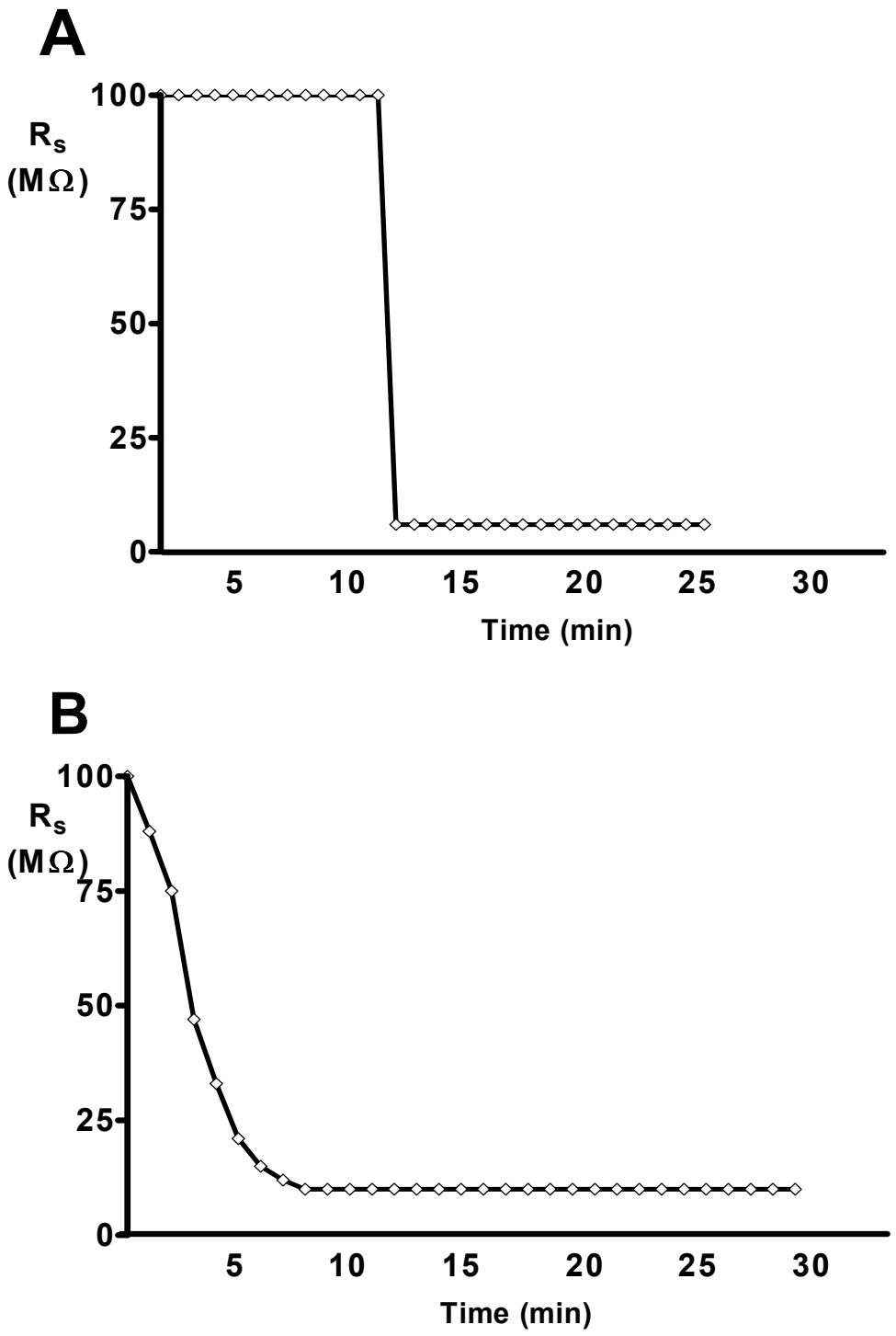


Figure 3.2 Representative examples of the time course of electrical access to the cell.
A Nystatin ruptured patch (NR), in 29% of myocytes there was no drop in Rs prior to electrical access (n= 74 cells from 39 patients).
B Nystatin perforated patch (NP), in 71% of myocytes Rs compensation was constantly adjusted during pore formation prior to electrical access (n= 181 cells from 60 patients).

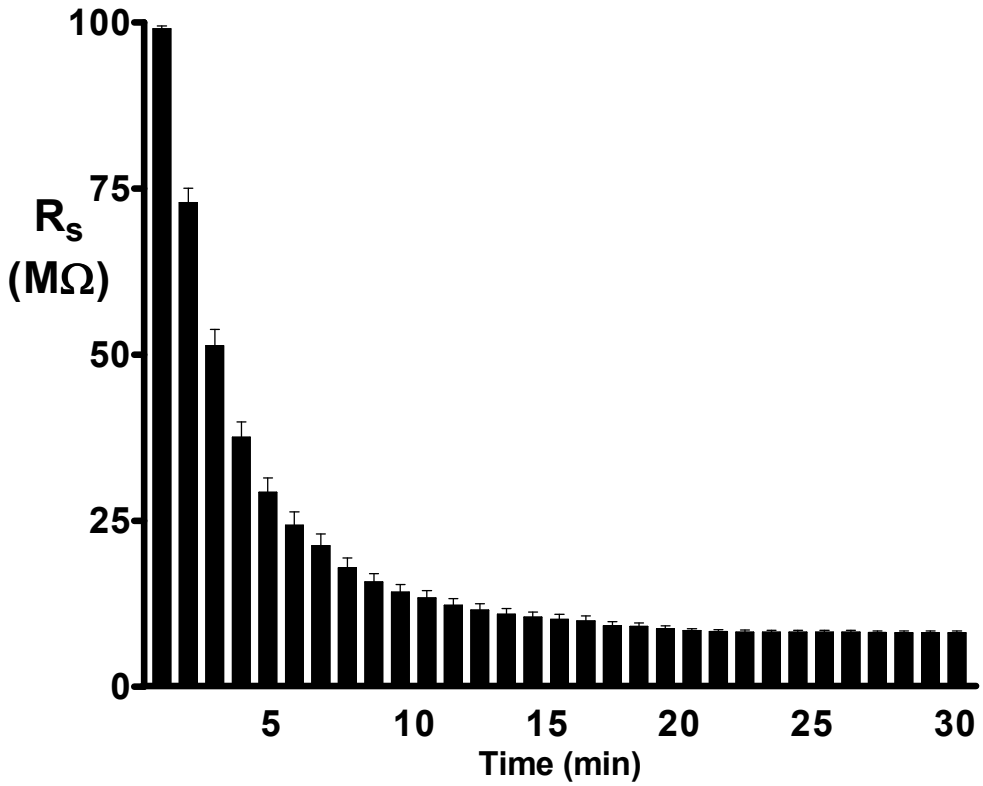


Figure 3.3 The mean time course of the formation of nystatin perforated patch electrical access to atrial myocytes as indicated by the falling electrical resistance (R_s) with time mean ± s.e.m. (n= 165 cells from 55 patients).

3.3 Protocols

Recordings of I_{CaL} were made using both the stepwise voltage pulse protocol to determine the current:voltage (IV) relationship and the repetitive time course peak I_{CaL} protocol as described in Methods section 2.4.1.. Figure 3.1B illustrates a typical recording of I_{CaL} under control conditions using the stepwise voltage pulse protocol. Following a voltage step from the holding potential (-40 mV) in incremental +10 mV depolarising pulses of 350 ms duration at 0.2 Hz, the cell and the superfusate were maintained at 37°C, a rapid and reproducible increase in inward calcium current occurred which reached a maximum in less than 5 ms and which appeared to decay in a bi-exponential fashion (Figure 3.1B) consistent with previous reports of human atrial I_{CaL}[161].

3.4 Statistics & data analysis

Data were recorded and analysed off-line as described in Methods section 2.5.1.. Subtraction of the steady state current at the end of the depolarising step from the peak inward current permitted estimation of the absolute magnitude of I_{CaL} for any given record. In order to assess the effect of ISO and ET-1 on the time constants of I_{CaL} inactivation the WCP traces were scaled and transposed into pCLAMP version 10 software Clampfit data analysis tables (Axon Instruments).

In order to determine the most appropriate statistical tests to employ, the distribution of values of peak I_{CaL} was assessed. As can be seen from Figure 3.4 the distribution of the direct observations of I_{CaL} current density was not Gaussian, with a leftward skew. However after logarithmic transformation of I_{CaL} current density the data did have a normal distribution and, therefore, the unpaired Student's t test was employed to determine the degree of difference between sub-analysed samples of I_{CaL} under control conditions. This test was selected as it is the most valid test with which to assess the probability of a similar or more extreme result being arrived at by chance in samples of small size. This precedent has been set in other published work examining the electrophysiology of human atrial myocytes[79;144;161;223]. Where a comparison between two differing populations of data are presented the variance of the two groups has been assessed to permit the use of the unpaired Student's t test, and if paired data are being compared the distribution of the variable being assessed was recorded and the paired Students' t test was employed. This report is based on all available information in order to maximise utility. In a small number of cases incomplete data was collected from patient records, comprising <3% data points, resulting in small variability in sample size depending on analysis undertaken.

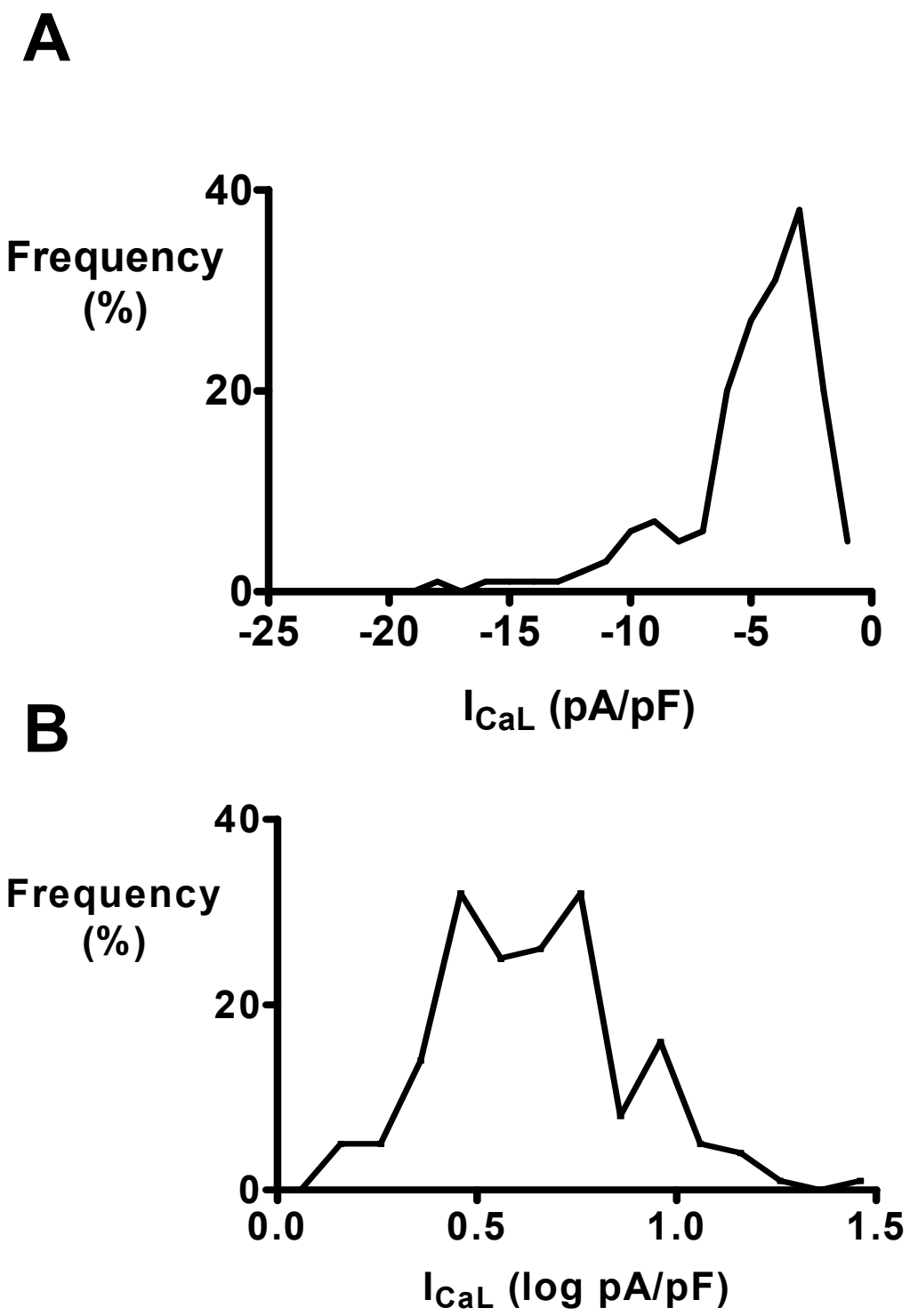


Figure 3.4 Frequency distribution graphs of peak I_{CaL} (n= 176 cells from 49 patients).
A Observed peak I_{CaL} current density under control conditions
B Logarithmic transformation of the magnitude of peak I_{CaL} current density under control conditions.

3.5 Results

3.5.1 Patients' clinical characteristics and methodological considerations

Clinical characteristics of all patients from whom samples of right atrial appendage were obtained are illustrated in Table 3.1. Post operative Atrial Fibrillation (AF_{pcs}) occurred in 19 of 65 patients (29%) within 7 days. None of the patients' clinical characteristics were significantly associated, by univariate analysis, with the development of AF_{pcs} and the data is presented in Table 3.2.

Cells from patients who were receiving ACE-inhibitor (ACE-I) therapy pre-operatively were associated with a significantly greater R_s, 7.66± 0.36 MΩ (n= 114 cells from 32 patients, mean± s.e.m.) as compared with those from patients who were not receiving ACE-I therapy, 6.73± 0.28 MΩ (n=130 cells from 33 patients, mean± s.e.m., $p< 0.05$). There were no other significant associations between patients' clinical characteristics and R_s C nor V_m demonstrated.

While there were no significant univariate associations between patients' clinical characteristics and mode of electrical access (Table 3.3), it can be seen from Figure 3.5 that fewer periods of enzymatic incubation of cells and greater myocyte capacitance are associated with successful perforated patch recordings in the absence of any significant effect on V_m (NR myocytes 75.3± 2.6 pF n=74 cells from 39 patients v. NP myocytes 86.4± 2.3 pF n=181 cells from 63 patients, mean± s.e.m., $p< 0.05$, Figure 3.5).

		n_p	%
Patient demographics	Male	52	80
	Age (years)	63.9± 1.2	
	Female	13	20
	Age (years)	67.3± 2.2	
Drug Therapy	Beta-Blocker	48	72
	Calcium Channel Blocker	21	32
	ACE- Inhibitor	32	49
Operative Procedure	CABG	58	89
	AVR	6	9
	CABG & AVR	1	2
LVSD	None (Normal)	32	50
	Mild	12	19
	Moderate	16	25
	Severe	4	6
Comorbidity	Hypertension	37	58
	Previous MI	33	51

Table 3.1 Patients' clinical characteristics.

		Post-op AF yes np (%)	Post-op AF no np (%)
Patient demographics	Male	13 (25)	39 (75)
	Female	6 (46)	7 (54)
	Age <75 (years)	14 (25)	42 (75)
	Age ≥75 (years)	5 (56)	4 (44)
	Pre-op HR <60 (bpm)	10 (33)	20 (67)
	Pre-op HR ≥60 (bpm)	8 (24)	26 (76)
Drug Therapy	Beta-Blocker	13 (28)	34 (72)
	No Beta-Blocker	7 (39)	11 (61)
	Calcium Channel Blocker	5 (24)	16 (76)
	No CCB	14 (32)	30 (68)
	ACE- Inhibitor	8 (25)	24 (75)
	No ACE- Inhibitor	11 (33)	22 (67)
Operative Procedure	CABG	15 (26)	43 (74)
	AVR	3 (50)	3 (50)
LVSD	Mild or None	12 (27)	32 (73)
	Moderate or Severe	6 (30)	14 (70)
	Ischaemic LVSD	6 (30)	14 (70)
	Non-ischaemic LVSD	3 (30)	7 (70)
Comorbidity	Hypertension	11 (30)	26 (70)
	No Hypertension	8 (30)	19 (70)
	Previous MI	9 (27)	24 (73)
	No Previous MI	10 (31)	22 (69)

Table 3.2 Patients' clinical characteristics and univariate analysis for post-op AF.

		Ruptured Patch n_c (%)	Perforated Patch n_c (%)
Patient demographics	Male	53 (29)	130 (71)
	Female	20 (33)	41 (67)
	Age <75 (years)	65 (30)	150 (70)
	Age ≥75 (years)	9 (23)	31 (77)
Drug Therapy	Beta-Blocker	59 (33)	122 (67)
	No Beta-Blocker	15 (20)	59 (80)
	Calcium Channel Blocker	22 (28)	57 (72)
	No CCB	51 (31)	114 (69)
	ACE- Inhibitor	28 (25)	86 (75)
	No ACE- Inhibitor	45 (35)	85 (65)
Operative Procedure	CABG	67 (32)	145 (68)
	AVR	4 (15)	23 (85)
LVSD	Mild or None	53 (32)	111 (68)
	Moderate or Severe	20 (25)	60 (75)
	Ischaemic LVSD	18 (21)	68 (79)
	Non-ischaemic LVSD	15 (34)	29 (66)
Comorbidity	Hypertension	43 (31)	96 (69)
	No Hypertension	27 (27)	74 (73)
	Previous MI	30 (25)	92 (75)
	No Previous MI	43 (35)	79 (65)

Table 3.3 Patients' clinical characteristics and univariate analysis for mode of electrical access.

Perforated patch recordings (NP) were associated with larger series resistance R_s than those obtained with ruptured patch (NR 5.35± 0.28 MΩ n=74 cells from 39 patients v. NP pipettes 7.97± 0.26 MΩ n=181 cells from 63 patients, mean± s.e.m., $p< 0.05$, Figure 3.5).

Consistent with previous reports[202;221;223] nystatin perforations formed consistently over a period of thirty minutes, indeed in this study more rapidly than ruptured patch seals (time to NR access 768± 67 s n=39 cells from 24 patients v. NP 473± 25 s n=143 cells from 55 patients, mean± s.e.m., $p< 0.05$, Figure 3.6). Perforated patch recordings were associated with more stable I_{CaL} recordings of longer duration as compared to ruptured patch recordings (NR I_{CaL} duration 458± 46 s n=48 cells from 26 patients v. NP 657± 39 s n=103 cells from 40 patients, mean± s.e.m., $p< 0.05$, Figure 3.6).

3.5.2 I_{CaL} kinetics

The I_{CaL} activation time was 4.1± 0.12 ms under control conditions (n= 7 cells from 7 patients). The I_{CaL} inactivation data were confirmed to fit a bi-exponential relation with mean time constants of tau_f (rapid phase time constant) = 11.8± 2.1 ms, tau_s (slow phase time constant)= 57.2± 5.7 ms n= 7 cells from 7 patients (Figures 3.7 and 3.8).

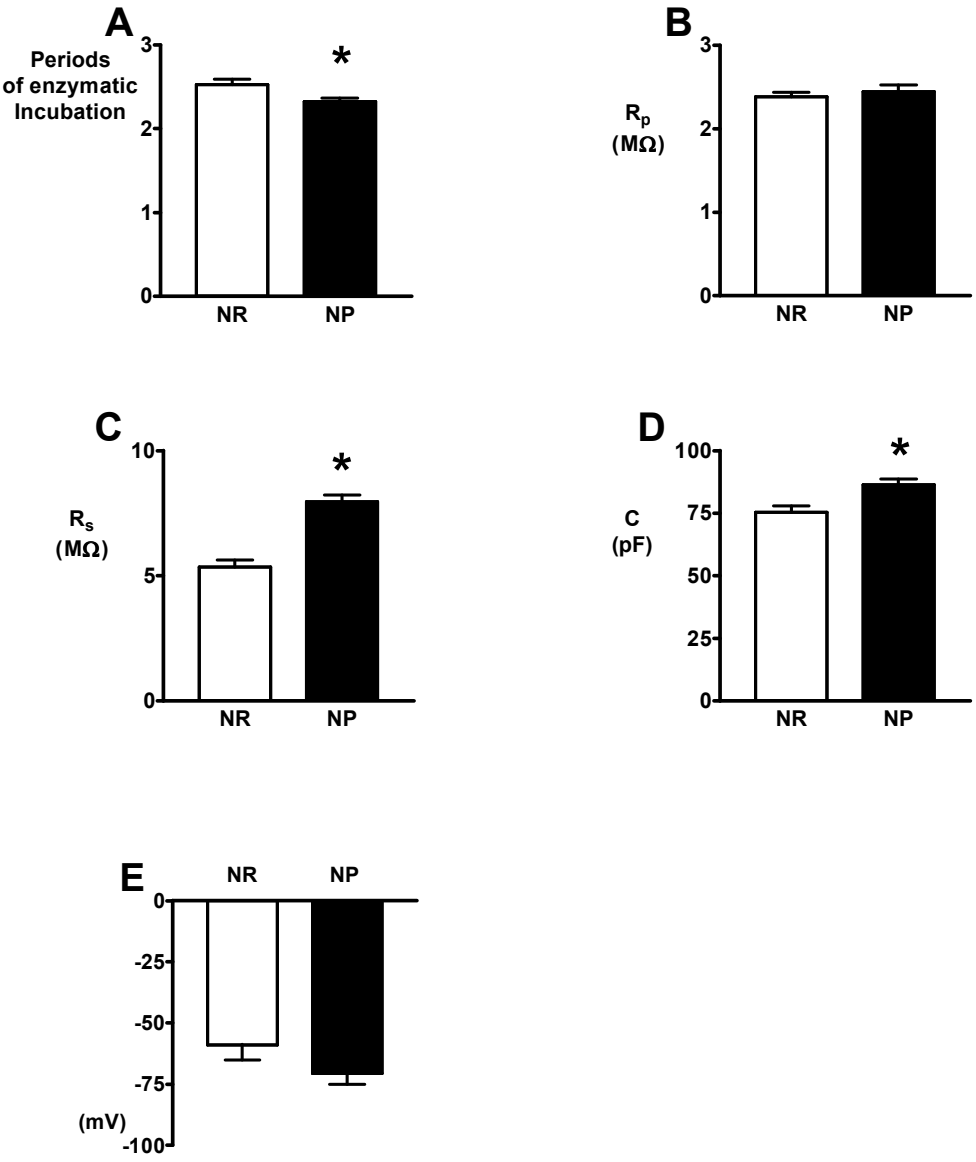


Figure 3.5 The possible interactions between methodology and cellular characteristics on mode of seal formation. □ = ruptured patch nystatin present in pipette (NR) ■ = perforated patch nystatin present in pipette (NP) Values are mean± s.e.m..

A Effect of increasing 10 minute periods of enzymatic incubation on the mechanism of electrical access to the cell. (NR n= 74 cells from 39 patients, NP n= 174 cells from 60 patients) $p< 0.05$. χ^2 test for incidence of NP access in cells from incubation 2 rather than incubation 1 or 3 $p<0.05$.

B Lack of effect of pipette resistance on mode of seal formation. (NR n= 49 cells from 25 patients, NP n= 50 cells from 28 patients).

C Effect of mode of seal formation on steady state series resistance. (NR n= 74 cells from 39 patients, NP n= 181 cells from 63 patients) $p< 0.05$.

D Effect of cell capacitance on mode of electrical access. (NR n= 74 cells from 39 patients, NP n= 181 cells from 63 patients) $p< 0.05$.

E Lack of effect of mode of seal formation on resting membrane potential (NR n= 13 cells from 9 patients, NP n= 68 cells from 20 patients).

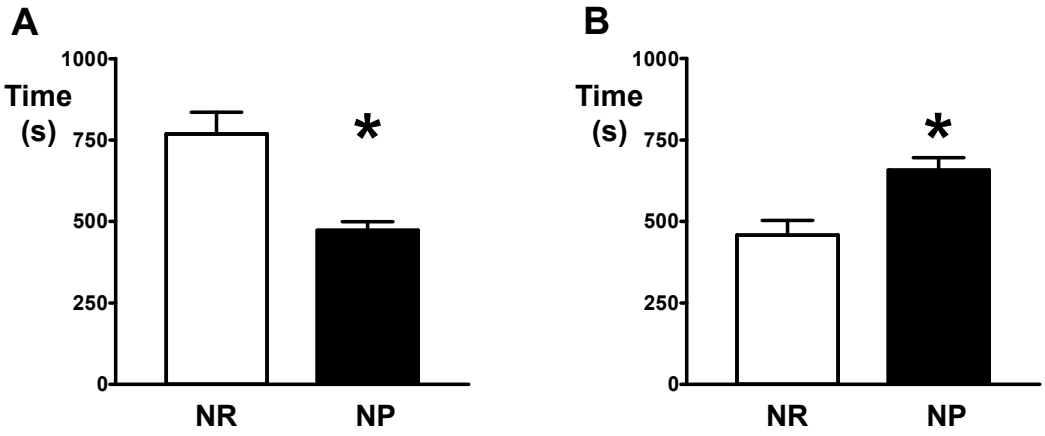


Figure 3.6 Effect of the mechanism of electrical access to the myocyte on duration of experiment. □ = ruptured patch nystatin present in pipette (NR) ■ = perforated patch Nystatin present in pipette (NP) Values are mean± s.e.m..
A Effect of the mechanism of electrical access to the myocyte on time taken to form electrical seal in seconds (NR n= 39 cells from 24 patients, NP n= 143 cells from 55 patients) $p< 0.05$.
B Effect of method of electrical access on the duration of stable I_{CaL} recordings in seconds.
(NR n= 48 cells from 26 patients, NP n= 103 cells from 40 patients) $p< 0.05$.

3.5.3 The current: voltage relationship of I_{CaL}

The mean current: voltage relationship of inward current recorded under control conditions at physiological temperature was a characteristic bell shaped curve as shown in Figure 3.9A. At potentials more negative than -30 mV and more positive than +40 mV little inward current was detectable. Peak inward current was recorded at +10 mV with a mean current density of -4.8± 0.4 pA/pF (n= 89 cells from 29 patients, mean± s.e.m.) corresponding with previous reports of I_{CaL}[161;163].

3.5.4 I_{CaL} peak current density

The peak current density, recorded at +10 mV, was noted in all cells in which I_{CaL} was measured. The mean peak current density from all cells (Figure 3.9B) was -5.1± 0.3 pA/pF (n= 176 cells from 49 patients, mean± s.e.m.) consistent with previously published work from this laboratory and others[79;144;161;223]. The mean peak I_{CaL} per patient or 'patient' mean was also calculated to be -4.8± 0.4 pA/pF

(n= 49 patients, mean± s.e.m.) and there was no significant difference between the 'cell' mean peak I_{CaL} and the 'patient' mean peak I_{CaL}(Figure 3.9B). Unless specifically stated to the contrary all mean values will refer to cell mean.

3.5.5 The time course of I_{CaL} rundown under control conditions

Peak I_{CaL} was recorded repetitively under control conditions in order to ascertain the degree of initial rundown (Figure 3.10). Cells which demonstrated irreversible rundown under control conditions (Figure 3.10B) were not immediately excluded from initial analysis in order to examine whether this property of cells could be predicted. Fortunately the number of cells which demonstrated irreversible rundown during control was only 12 from 188 recordings (6.4%). The vast majority of these recordings were made in the initial stages of the project, eleven in the first twenty recordings consistent with the author's learning curve. There were no significant differences in the cellular characteristics of those cells exhibiting irreversible rundown. Electrophysiological data relating to these cells is not incorporated further into this thesis.

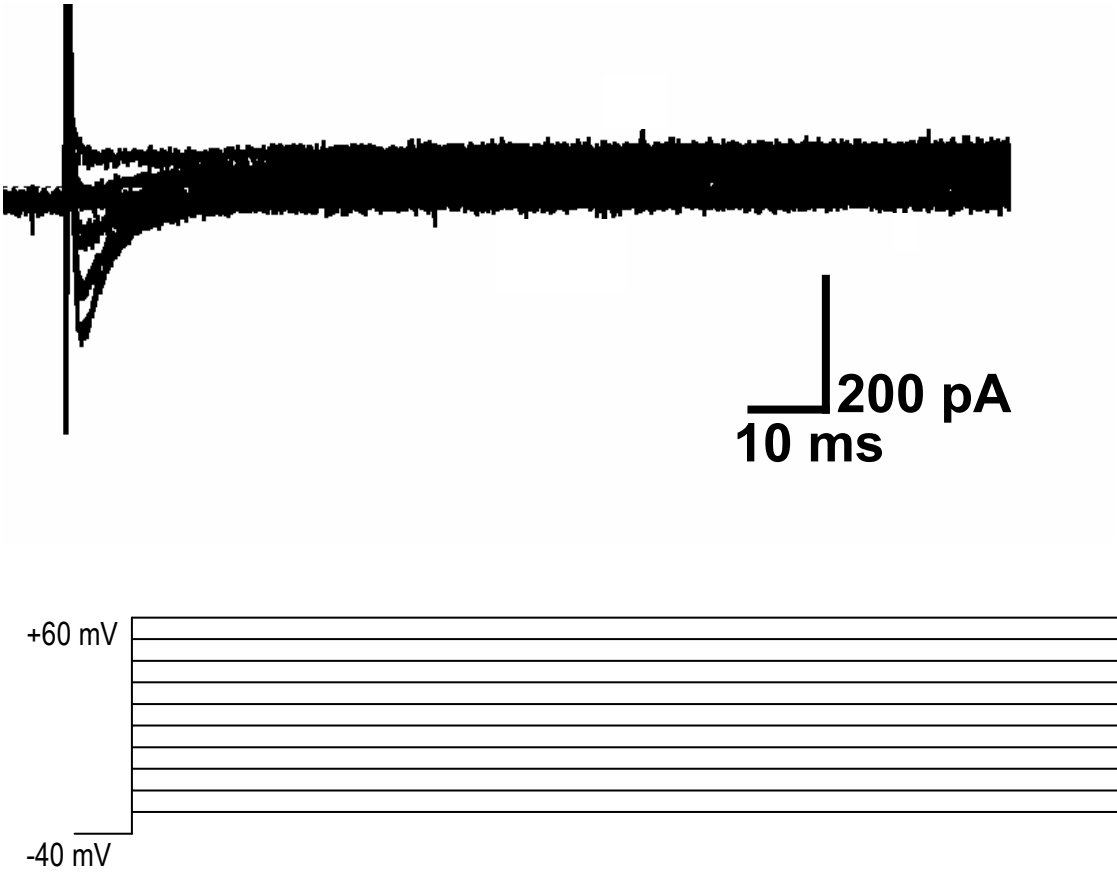


Figure 3.7 An example of a raw inward current trace (top) obtained using the voltage pulse protocol (lower) under control conditions with stepwise depolarisations, increasing in 10 mV increments from a holding potential of -40 mV to a maximum of +60 mV demonstrating the rapid inward current with biexponential decay.

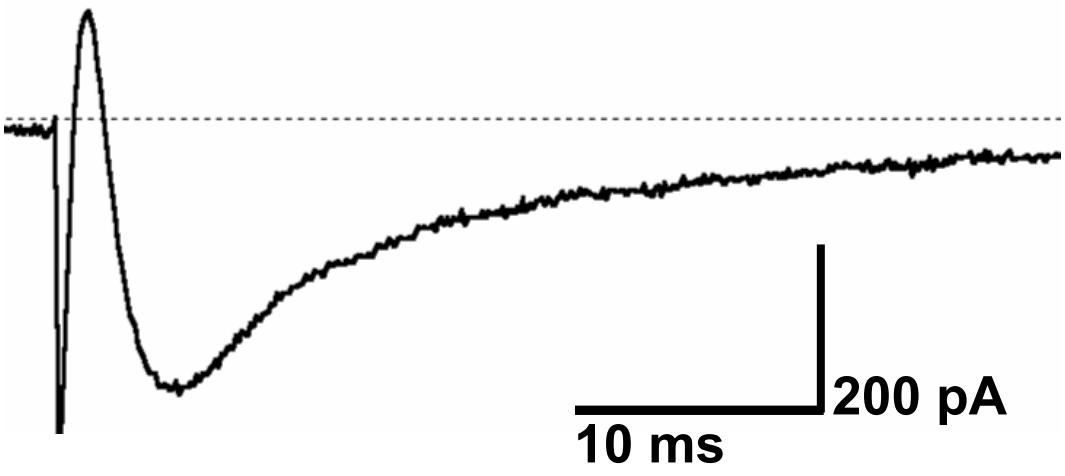


Figure 3.8 Representative example of peak I_{CaL} under control conditions demonstrating rapid activation and biexponential decay of I_{CaL} (tau_f = 11.9± 1.4 ms, tau_s = 57.4± 2.9 ms)
Mean values± s.e.m. tau_f = 11.8± 2.1 ms, tau_s = 57.2± 5.7 ms (n= 7 cells from 7 patients).

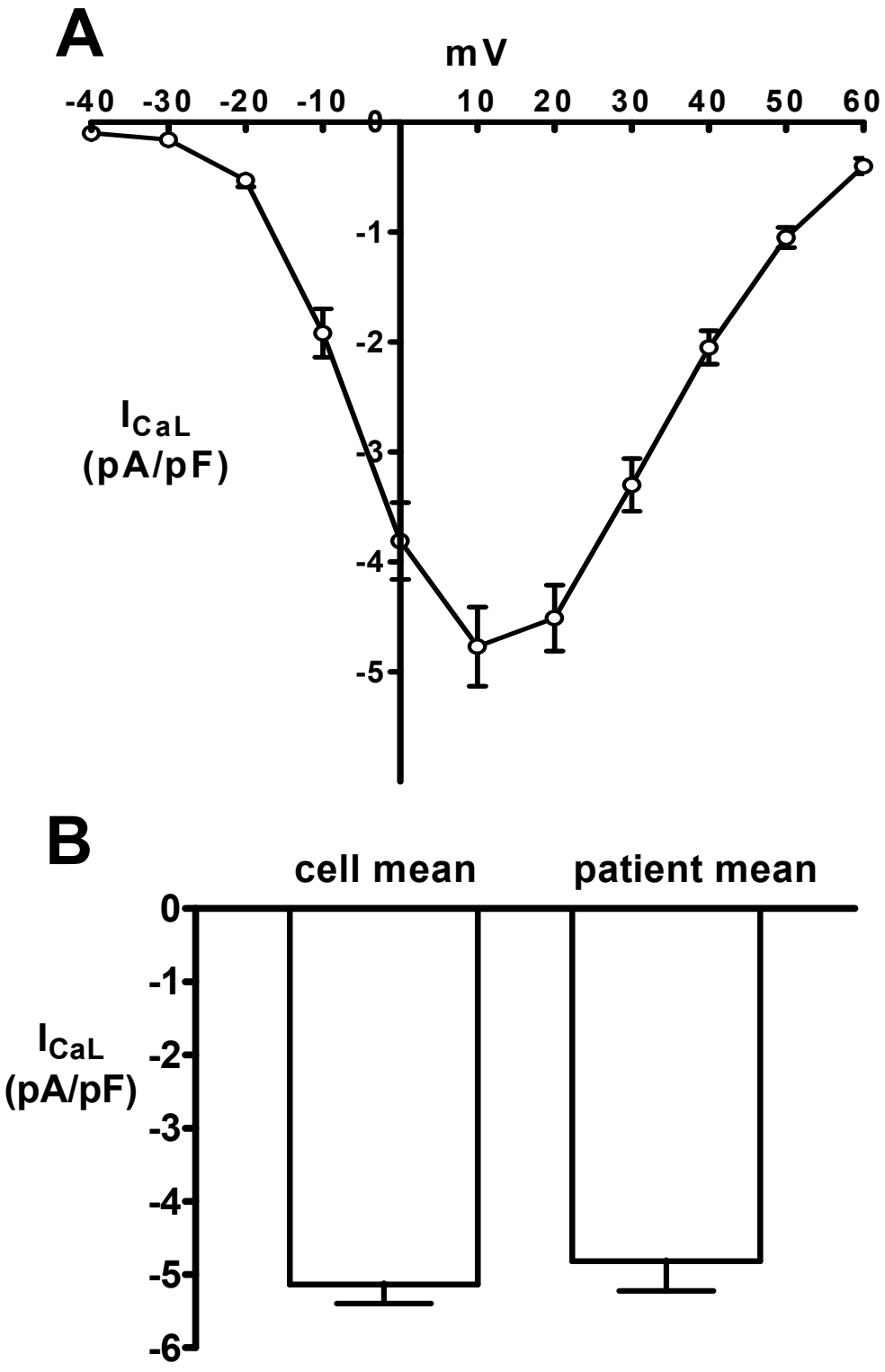


Figure 3.9 The I:V relationship of I_{CaL} and comparison of cell v. patient mean.
A The mean current:voltage relationship obtained in all cells (n= 89 cells from 29 patients mean± s.e.m.).
B Comparison of 'cell' mean v 'patient' mean peak current density of I_{CaL} in all cells (n= 176 cells from 49 patients, mean± s.e.m.)

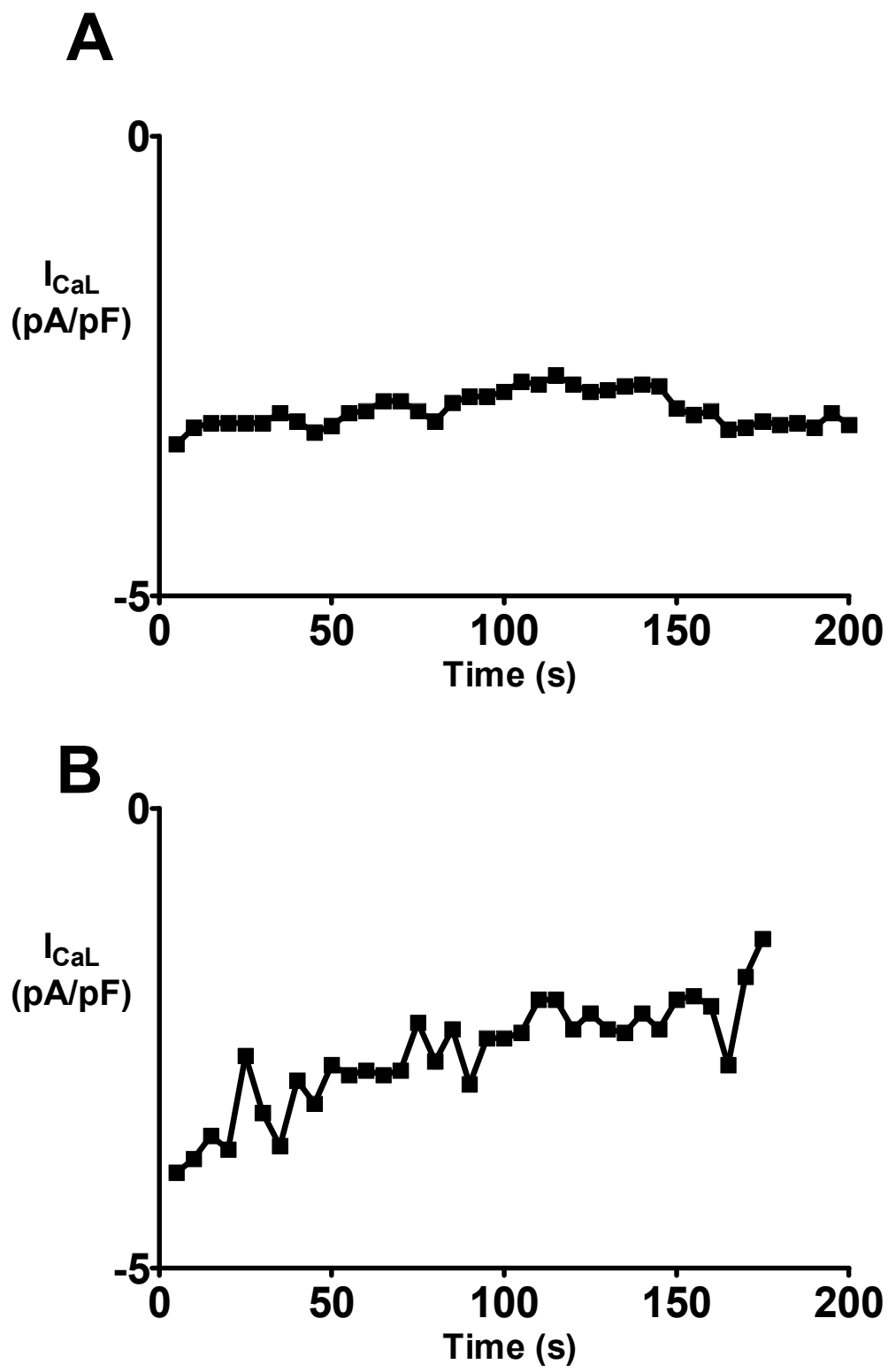


Figure 3.10 Comparison of stable I_{CaL} recordings with 'run down'.
A Example of stable I_{CaL} under control conditions with 'permissible' rundown with time.
B Example of unstable I_{CaL} under control conditions with 'unacceptable' rundown with time.

3.5.6 Impact of the method of seal formation on I_{CaL}

There was no significant difference (Figure 3.11) in the I:V relationship of I_{CaL} in those myocytes in which the seal was obtained via a ruptured patch as compared with those cells in which the seal was a perforated patch. There was no significant difference in the magnitude of peak I_{CaL} in those myocytes in which the seal was obtained via a ruptured patch -4.7± 0.4 pA/pF (n= 65 cells from 31 patients, mean± s.e.m.) as compared with those cells in which the seal was a perforated patch, -5.4± 0.4 pA/pF (n=111 cells from 41 patients, mean± s.e.m., Figure 3.12A). Figure 3.18B illustrates that there was no significant difference in the variance between the two groups and therefore the Students' t test is a valid test to compare the two groups.

3.5.7 Impact of patients' clinical characteristics on I_{CaL}

3.5.7.1 Patient age and I_{CaL} characteristics

The mean I: V relationship of I_{CaL} recorded from myocytes isolated from patients aged 75 years or older was found to be similar to that obtained from patients aged younger than 75 years (Myocytes from patients aged ≥75 years n= 19 cells from 6 patients, v. Myocytes from patients aged <75 years n= 70 cells from 23 patients, mean ± s.e.m.). Mean peak I_{CaL} under control conditions was not significantly different between myocytes isolated from patients aged ≥75 years and that from younger patients (Myocytes from patients aged ≥75 years peak I_{CaL} -5.2± 0.8 pA/pF, n= 33 cells from 8 patients, v. Myocytes from patients aged <75 years peak I_{CaL} -5.1± 0.3 pA/pF, n= 143 cells from 41 patients, mean± s.e.m.).

3.5.7.2 Gender and I_{CaL} characteristics

There was no significant difference in the current: voltage relationship of I_{CaL} in those myocytes obtained from male as compared with female patients. There was no significant difference in peak I_{CaL} in those myocytes obtained from male patients when compared with myocytes obtained from female patients. (Male cells -5.3± 0.3 pA/pF, n= 143 cells from 40 patients, v. Female cells -4.5± 0.5 pA/pF, n= 33 cells from 9 patients, mean± s.e.m.).

3.5.8 Impact of chronic pre-operative beta-adrenoceptor blocking (β-B) therapy on I_{CaL}

3.5.8.1 Pre-operative heart rate and β-B therapy

All 48 patients (72% of total) received beta-adrenoceptor blocking drugs (beta-blockers, β-B) continuously for a minimum of 30 days pre-operatively until the peri-operative period. The most common agent used was Atenolol (39 patients, 81%) in dosages ranging from 25-75 mg daily (mean dose 41 mg, mode 50 mg). Other agents were Bisoprolol (4 patients, 9%), Metoprolol (3 patients, 6%) and Carvedilol (2 patients 4%). No patient was receiving Sotalol. The preoperative heart rate as recorded on the 12 lead electrocardiogram was significantly lower in those patients receiving beta blockers (BBY 59± 1 bpm v BBN 76± 1 bpm, n= 48 and 16 patients respectively $P< 0.05$).

3.5.8.2 β-B therapy and I_{CaL} characteristics

There was no significant difference in the current: voltage relationship nor peak I_{CaL} in those myocytes obtained from patients who had, as opposed to had not, received chronic pre-operative β-B therapy (I: V relationship Figure 3.21A: peak I_{CaL}: BBY -5.4± 0.3 pA/pF, n= 124 cells from 36 patients, v. BBN -4.5± 0.4 pA/pF, n=52 cells from 13 patients, mean± s.e.m.).

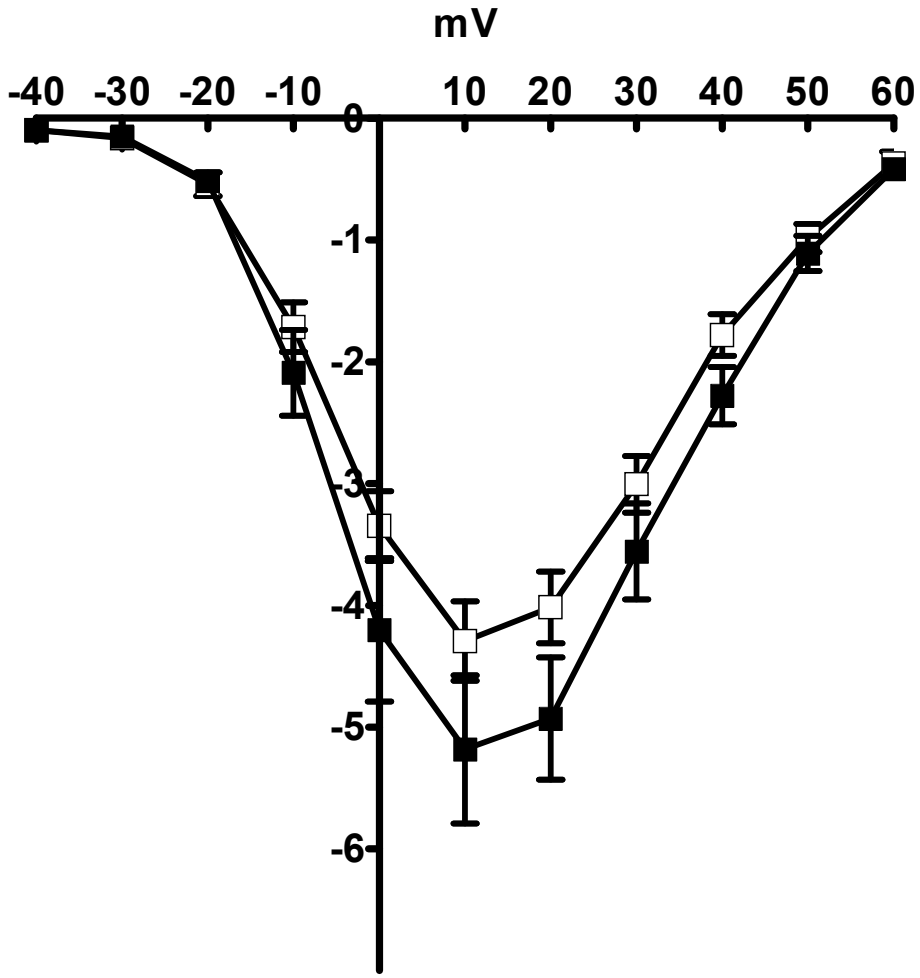


Figure 3.11 Lack of effect of method of electrical access on L-type calcium current (I_{CaL}) current:voltage relationship under control conditions. □ = ruptured patch nystatin present in pipette (NR) (n= 41 cells from 21 patients) ■ = perforated patch Nystatin present in pipette (NP) (n= 48 cells from 23 patients). Values are mean± s.e.m.

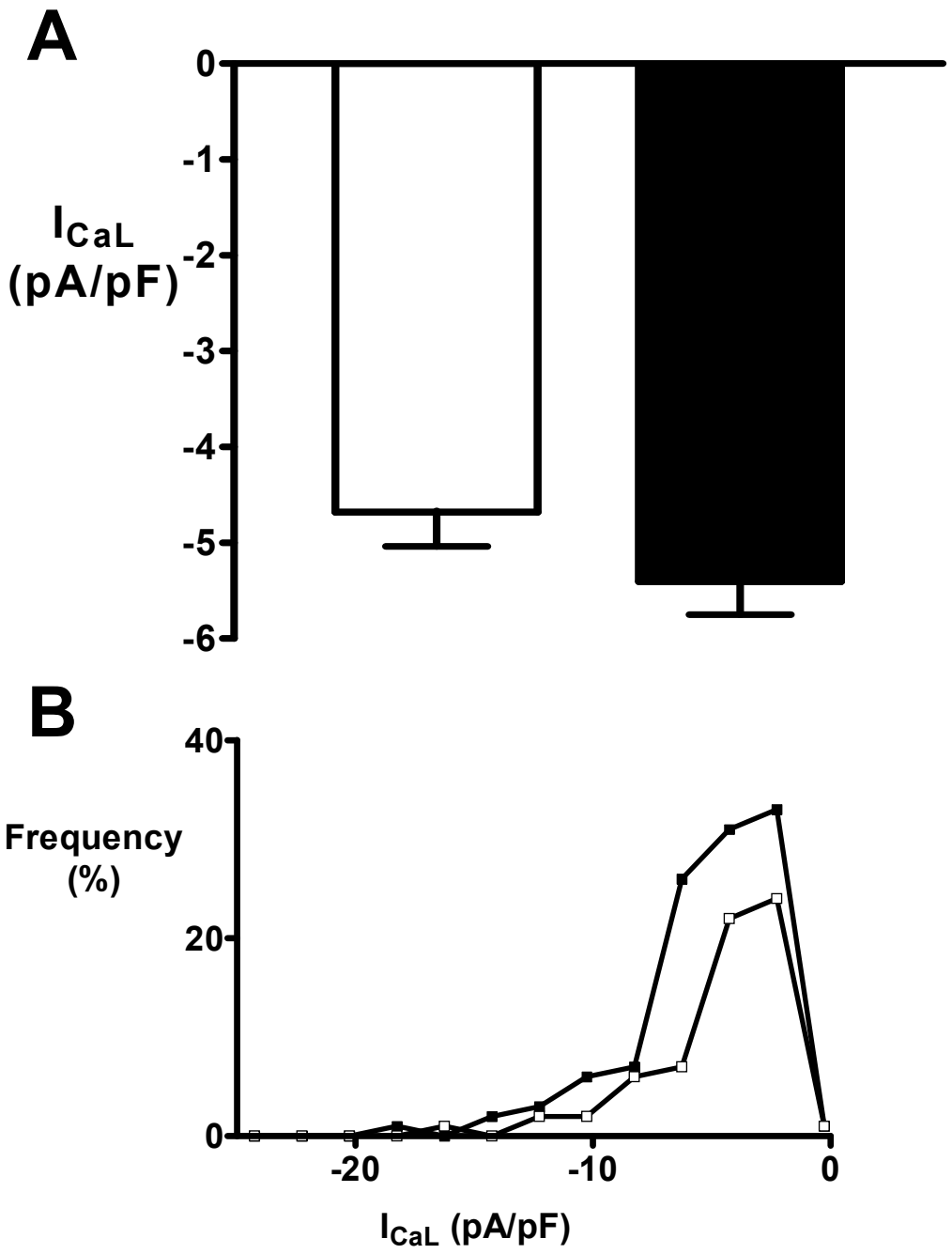


Figure 3.12 Comparison of mode of electrical access and peak I_{CaL}. □ = ruptured patch nystatin present in pipette (NR) (n= 65 cells from 31 patients) ■ = perforated patch Nystatin present in pipette (NP) (n= 111 cells from 41 patients). Values are mean± s.e.m.
A Lack of effect of method of electrical access on peak (measured at +10 mV) L-type calcium current (I_{CaL}) density under control conditions.
B Lack of significant difference in variance in the distribution of peak I_{CaL} recordings related to method of electrical access.

3.5.9 Impact of chronic pre-operative calcium channel blocker (CCB) therapy on I_{CaL}

3.5.9.1 Pre-operative heart rate and CCB therapy

All 21 patients (32% of total) received calcium channel blocking drugs (CCB) continuously for a minimum of 3 months pre-operatively and throughout the peri-operative period. The most common agent used was Amlodipine (15 patients, 72%) at either 5 or 10 mg. One patient was receiving Nifedipine. The remaining 5 patients (24%) were prescribed Diltiazem betweem 60 and 300 mg. No patients were prescribed Verapamil. The preoperative heart rate as recorded on the 12 lead electrocardiogram was not significantly different in those patients receiving calcium channel blocking drugs from those not receiving CCB drugs pre-operatively (CCBY 62± 2 bpm v CCBN 64± 1 bpm, n= 21 and 43 patients respectively).

3.5.9.2 CCB therapy and I_{CaL} characteristics

There was no significant difference in either the current: voltage relationship or peak I_{CaL} in those myocytes obtained from patients who had received chronic calcium channel blocking therapy pre-operatively (CCBY -4.7± 0.4 pA/pF, n= 55 cells from 17 patients, v. CCBN -5.4± 0.3 pA/pF, n=121 cells from 32 patients, mean± s.e.m.).

3.5.10 The impact of chronic Angiotensin Converting Enzyme Inhibitor (ACE-I) therapy on I_{CaL}

All 32 patients (49% of total) received ACE-I medications continuously for a minimum of 3 months pre-operatively until the peri-operative period. In 11 patients (34%) this medication was withheld on the last preoperative day. The most common agent used was Ramipril (20 patients, 63%) in dosages ranging from 2.5-10 mg daily (mean dose 7.7 mg, mode 10 mg). Other agents were Lisinopril (8

patients, 25%), Enalapril (2 patients, 6%), Perindopril and Trandolapril (1 patient each 3%). There was no significant difference in the current: voltage relationship of I_{CaL} in those myocytes obtained from patients who had received chronic ACE-I therapy. There was no significant difference in peak I_{CaL} in those myocytes obtained from patients who had received chronic pre-operative ACE-I therapy when compared with myocytes obtained from patients who had not received preoperative ACE-I therapy (ACE-I Y -5.4± 0.5 pA/pF, n= 112 cells from 28 patients, v. ACE-I N -5.0± 0.3 pA/pF, n=64 cells from 21 patients, mean± s.e.m.).

3.5.11 Impact of pre-operative left ventricular systolic dysfunction (LVSD) on I_{CaL}

There was no significant difference in the current: voltage relationship between either normal compared with abnormal Left Ventricular Systolic Dysfunction (LVSD), as defined by pre-operative echocardiography, nor between the aggregated groups of Normal & Mild LVSD compared with Moderate & Severe LVSD. There was no significant difference between the peak current density in those cells from patients with normal compared to abnormal LVSD (Normal LV systolic function peak I_{CaL} -5.0± 0.4 pA/pF n= 77 cells from 23 patients, v. Abnormal LV systolic function peak I_{CaL} -5.1± 0.4 pA/pF n= 77 cells from 21 patients, Figure 3.13A). However the peak current density recorded from cells with moderate and severe LVSD was statistically significantly greater than that recorded from cells with normal or mild LVSD (Normal and Mild LVSD peak I_{CaL} -4.8± 0.3 pA/pF n= 108 cells from 32 patients, v. Moderate and Severe LVSD peak I_{CaL} -5.6± 0.6 pA/pF n= 46 cells from 12 patients $p< 0.05$, Figure 3.13B).

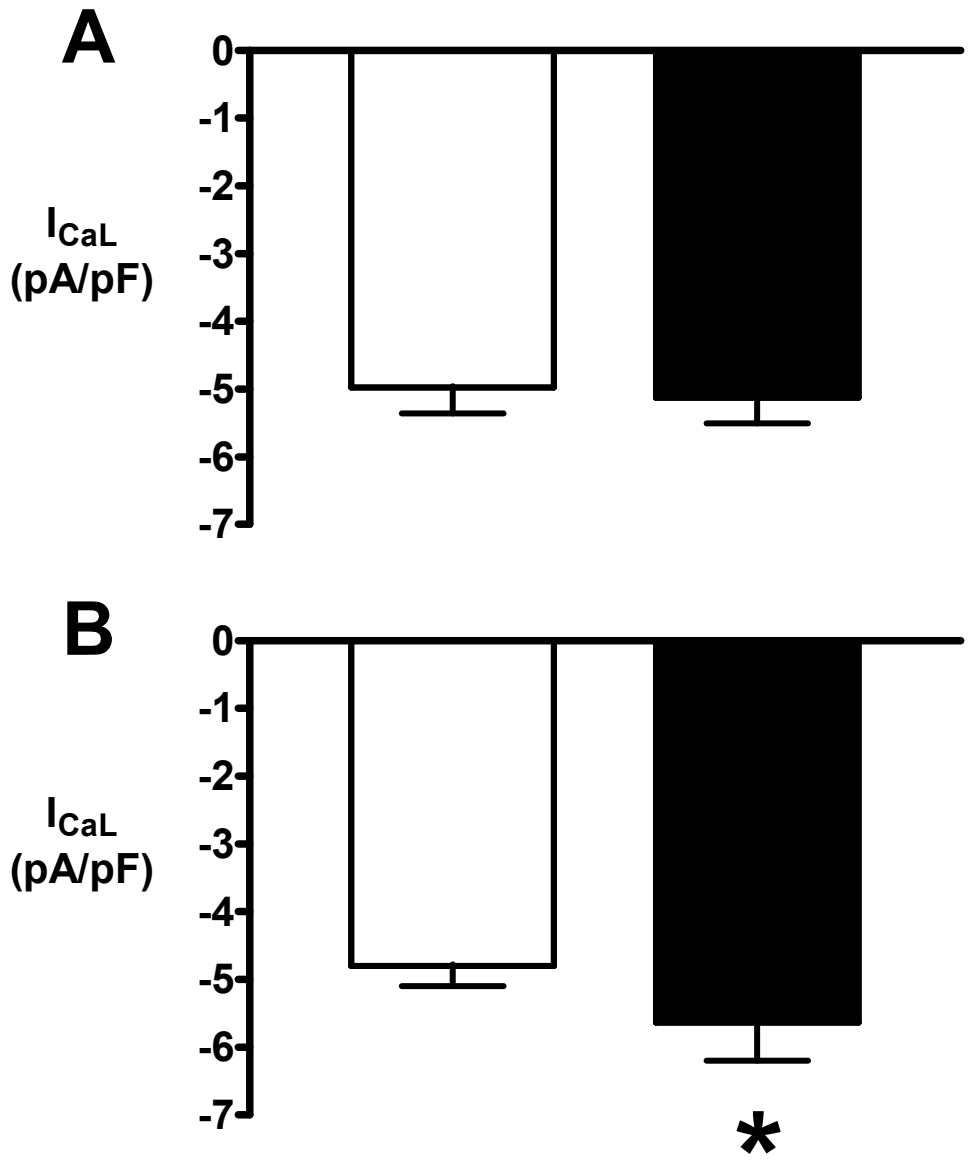


Figure 3.13 Impact of LVSD on peak I_{CaL}. Values are mean± s.e.m.
A Lack of effect of any degree of pre-operative Left Ventricular Systolic Dysfunction (LVSD) on peak I_{CaL} under control conditions. □ = patients without LVSD (n= 77 cells from 23 patients) ■ = patients with LVSD (n= 77 cells from 21 patients).
B Effect of moderate or severe pre-operative Left Ventricular Systolic Dysfunction (LVSD) on peak I_{CaL} under control conditions. □ = patients with mild or no LVSD (n= 108 cells from 32 patients) ■ = patients with moderate or severe LVSD (n= 46 cells from 12 patients). $p< 0.05$.

3.5.12 Impact of prior Myocardial Infarction on I_{CaL}

There was no significant difference in the current: voltage relationship between patients who had suffered a prior Myocardial Infarction (MI), as recorded in the clinical case sheets, and those patients who had not had a documented previous MI (Figure 3.14A). In contrast, myocytes isolated from patients who had suffered a documented MI pre-operatively had a significantly greater peak I_{CaL} current density on comparison with myocytes from patients who had not suffered a prior MI (MIN -4.52± 0.35 pA/pF, n= 83 cells from 23 patients v. MIY -5.55± 0.36 pA/pF, n= 88 cells from 25 patients, $p< 0.05$ Figure 3.14B).

3.5.13 Impact of aetiology of Left Ventricular Systolic Dysfunction (LVSD) on I_{CaL}

There was no significant difference in the current: voltage relationship between patients who had ischaemic cardiomyopathy (ICM) and those suffering non-ischaemic cardiomyopathy (NICM). However, I_{CaL} was of a significantly lower density from myocytes isolated from patients with NICM when depolarised to potentials more positive than zero. $p< 0.05$ (Figure 3.15A). Peak I_{CaL}, however, was of a significantly lower density from myocytes isolated from patients with NICM when compared to myocytes from patients with ischaemic cardiomyopathy (NICM -3.52± 0.3 pA/pF, n= 25 cells from 6 patients, ICM -5.87± 0.47 pA/pF, n= 62 cells from 17 patients, $p< 0.05$ Figure 3.15B).

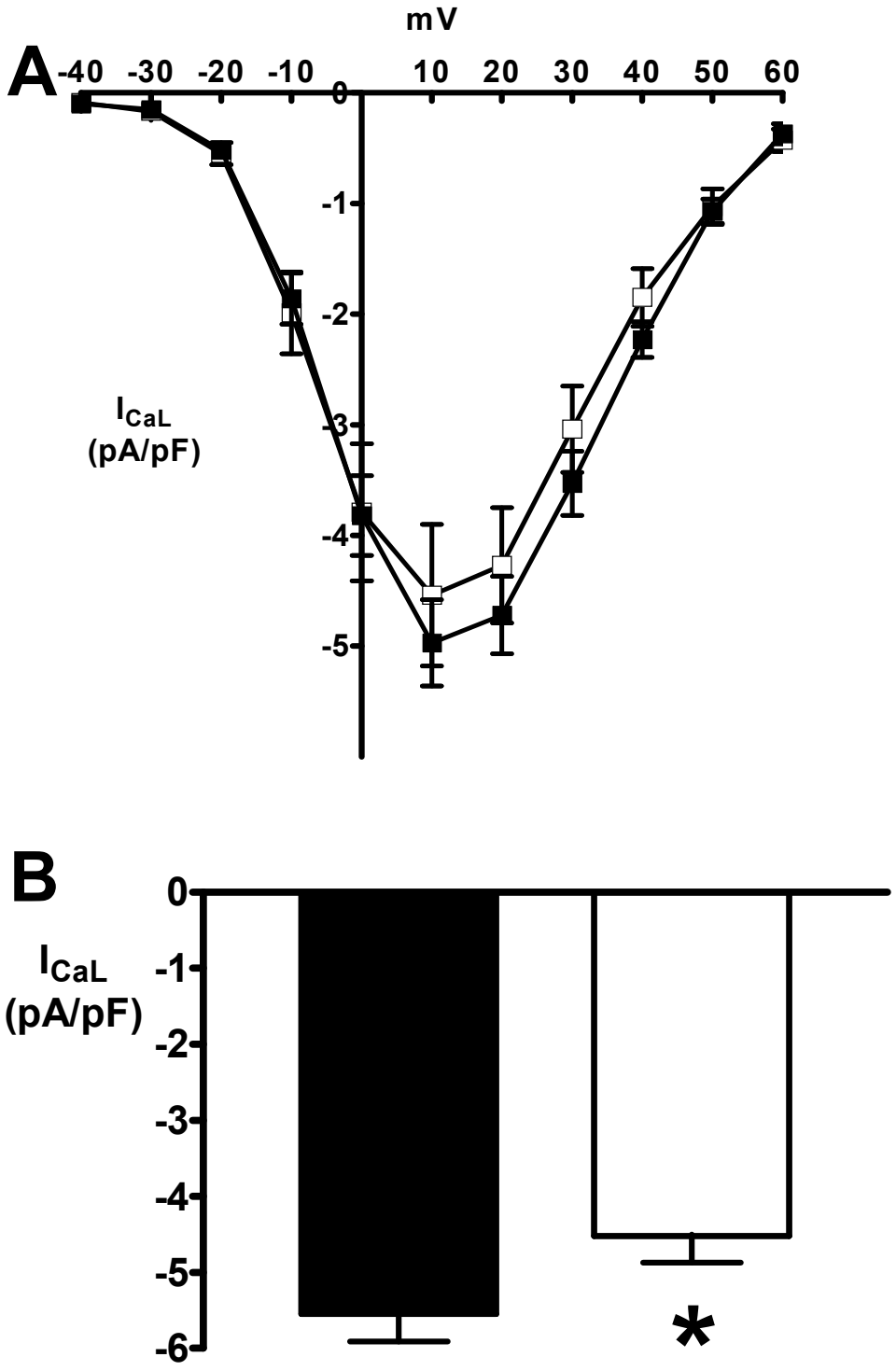


Figure 3.14 Impact of prior Myocardial Infarction (MI) on I_{CaL}. □ = patients who have not sustained previous MI (MIN) ■ = patients who have sustained previous MI (MIY) Values are mean± s.e.m..
A Lack of effect of prior MI on I_{CaL} I: V relationship under control conditions. (MIN n= 41 cells from 13 patients, MIY n= 48 cells from 16 patients).
B Effect of prior MI on peak I_{CaL} under control conditions (MIN n= 83 cells from 23 patients, MIY n= 88 cells from 25 patients) $p< 0.05$.

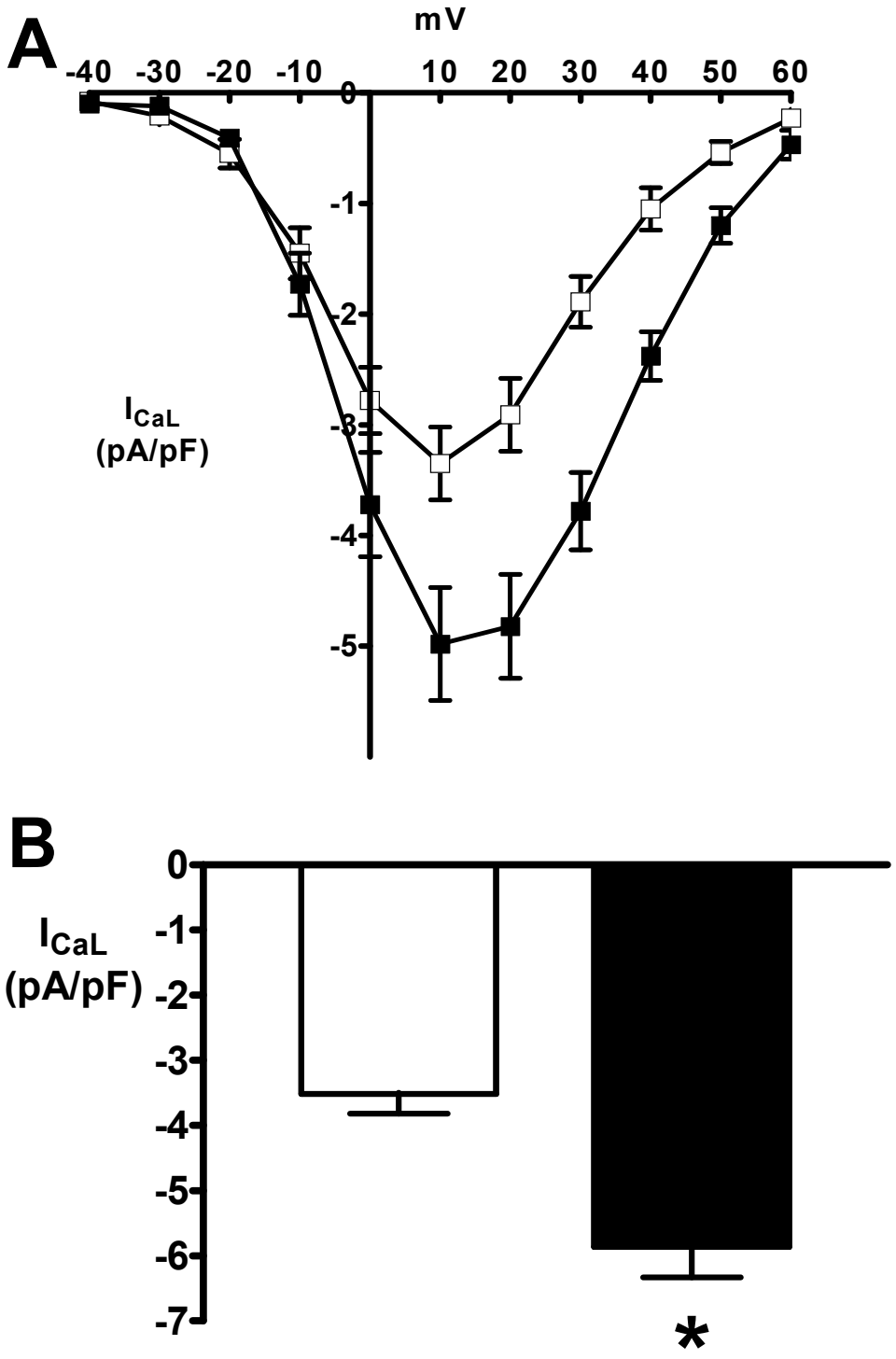


Figure 3.15 Impact of aetiology of LVSD on I_{CaL}. □ = patients who have non-ischaemic cardiomyopathy (NICM) ■ = patients who have ischaemic cardiomyopathy (ICM) Values are mean± s.e.m..

A Lack of effect of aetiology of LVSD on I_{CaL} I: V relationship under control conditions. (NICM n= 15 cells from 4 patients, ICM n= 28 cells from 10 patients).

B Effect of aetiology of LVSD on peak I_{CaL} under control conditions (NICM n= 25 cells from 6 patients, ICM n= 62 cells from 17 patients).$p< 0.05$.

3.5.14 The effect of ISO on I_{CaL} current: voltage relationship

Isoproterenol at 0.1 μM significantly increased the magnitude of the inward current without altering the current: voltage relationship of I_{CaL} (Figure 3.16A) at physiological temperature. (n= 4 cells from 3 patients, paired data, mean± s.e.m. p< 0.05.) An example of the paired peak (+10 mV) current traces is depicted in Figure 3.16B.

3.5.15 The effect of ISO on activation and inactivation of I_{CaL}

Isoproterenol at 0.05 μM had no significant effect on the activation time (control 4.13± 0.15 ms v. 4.3± 0.37 ms n= 4 cells from 4 patients) or time constant of inactivation of I_{CaL} (control 54.0± 10.1 ms v. 55.2± 14.8 ms n= 4 cells from 4 patients). Figure 3.16B is an example of raw current tracings demonstrating this lack of effect and mean data are presented in histograms in Figure 3.17.

3.5.16 The effect of ISO on peak I_{CaL}

3.5.16.1 Time course of ISO effect on peak (+10 mV) current density

There were four differing patterns of change in peak I_{CaL} following acute superfusion of ISO which we classified into four response 'types' (Figures 3.18 and 3.19). The type O response, in which there was no discernible effect of ISO on peak I_{CaL}, occurred in only 3% of cells (Figure 3.18A). Due to the combination of the rarity of this type of response, the early loss of seal, the subjective visible signs of cell death (shrinkage, spherication, crenation, loss of discernable striations) and the literature describing the consistent positive effect of ISO to increase I_{CaL}[81;94;114;144;155;161;199;200;221;225;279-282] these cells were excluded from further analysis.

The most common adrenergic response, designated the type 1 response, occurred in 60% of cells. This response was characterised by a stable augmented I_{CaL} current density persisting as a plateau on the time course curve-plot, see Figure 3.18B.

A type 2 response in which the initial increase in peak I_{CaL} current density was temporary but an intermediate plateau was reached which was of greater current density than that recorded under control conditions but less than the peak initial response occurred in 10% of cells (see figure 3.19A).

Finally a type 3 response to ISO was that of an initial increase in I_{CaL} which did not reach a plateau and peak I_{CaL} irreversibly ran down to control levels and occurred in 30% of cells (Figure 3.19B).

3.5.16.2 ISO effect on peak (+10 mV) current density

An example of the effect of acute superfusion with 0.05 μM ISO on the raw current tracings can be seen in Figure 3.20A. The significant mean increase in peak I_{CaL} following acute superfusion with 0.05 μM ISO in absolute terms can be seen in Figure 3.20 and the relative increase, that is the magnitude of the increase in current density expressed as a percentage of the current density under control conditions was 218.8± 17.4 % (n= 87 cells from 36 patients, paired data, mean± s.e.m. $p< 0.05$).

3.5.16.3 Concentration: response relationship of ISO on peak I_{CaL}

Isoproterenol, at concentrations equal to or less than 10^{-9} had no significant effect on the magnitude of peak I_{CaL} (+10 mV), however at all concentrations tested in the range from 10^{-8} to 10^{-6} M, acute superfusion with ISO significantly increased the magnitude of peak I_{CaL} (Figures 3.21 and 3.22).

An interim concentration: response curve of the maximal initial effects of ISO on peak I_{CaL} was constructed during the early stages of the project in order to select a suitable concentration of ISO for later experiments (Figure 3.22A). From this curve we calculated that the EC_{50} of ISO was approximately 0.05 μM ISO and that the maximal response was likely to occur at approximately 1.0 μM ISO.

As can be seen from the final concentration: response curve of the adrenergic effect of ISO on peak I_{CaL} (Figure 3.22B) the true EC_{50} of ISO was approximately 0.013 μM ISO, consistent with initial experience with the nystatin perforated patch clamp recordings of I_{CaL}[221].

3.5.16.4 Time to maximal ISO effect on peak (+10 mV) current density

The mean time to peak effect of ISO on I_{CaL} was 95± 4 seconds (n= 113 cells from 39 patients Figure 3.23A). There was no significant difference in the time to peak effect of increasing ISO concentration (Figure 3.23A). There was no significant difference in the time taken to peak ISO effect of either method of electrical access (Figure 3.23B). However, ISO at 0.05 μM, had a significantly more rapid peak effect on I_{CaL} in cells from patients who had received chronic pre-operative beta-adrenoceptor antagonists (Figure 3.23C).

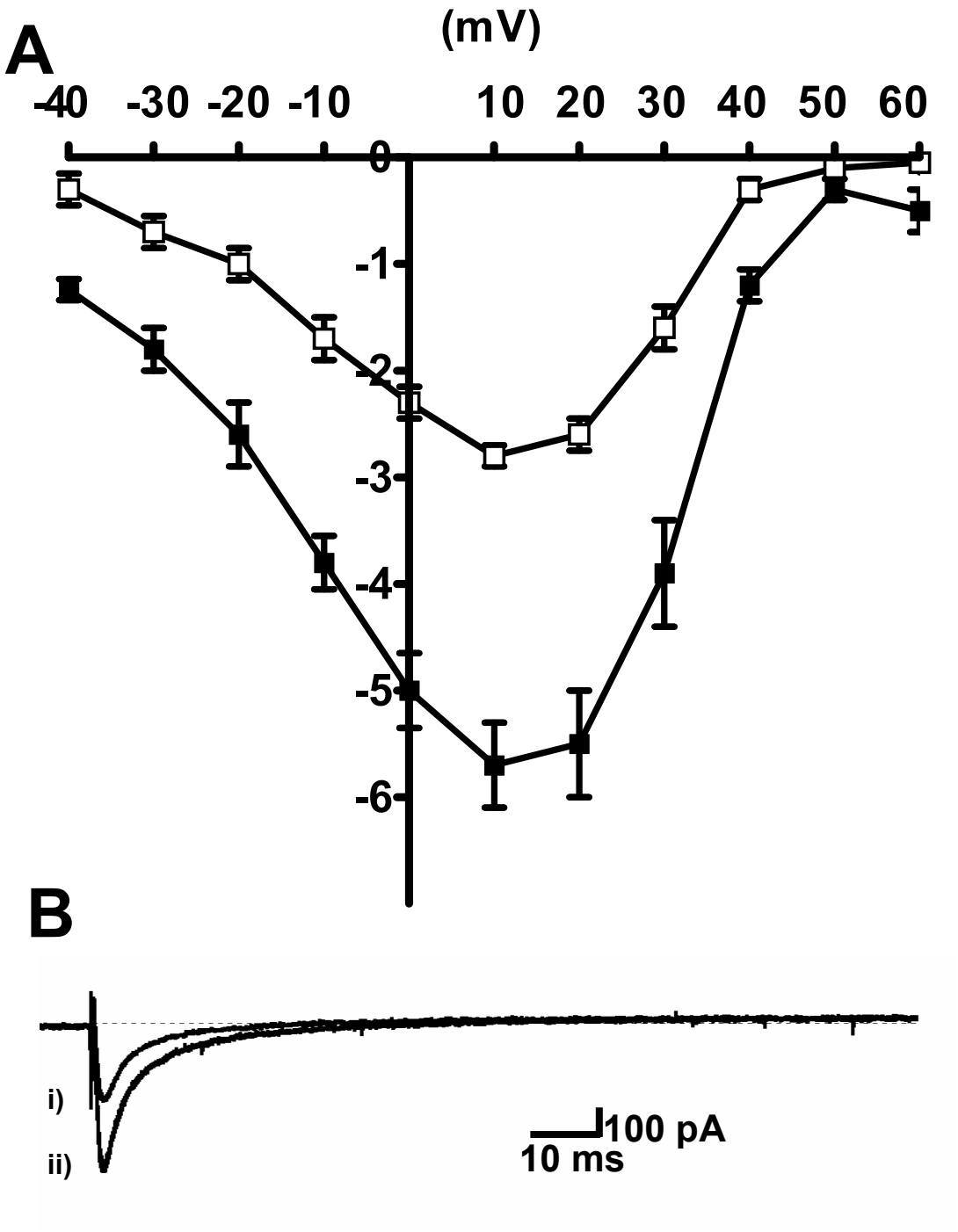


Figure 3.16 The effect of acute superfusion of ISO at 0.1 μM on of I_{CaL}.
A The effect of ISO 0.1 μM on the I: V relationship of I_{CaL}. □ = control ■ = ISO 0.1 μM ISO (n=4 cells from 3 patients, paired data, mean± s.e.m. $p< 0.05$.)
B Examples of two superimposed raw current tracings of peak I_{CaL} recorded from the same myocyte: i) under control conditions ii) following acute superfusion with ISO 0.1 μM.

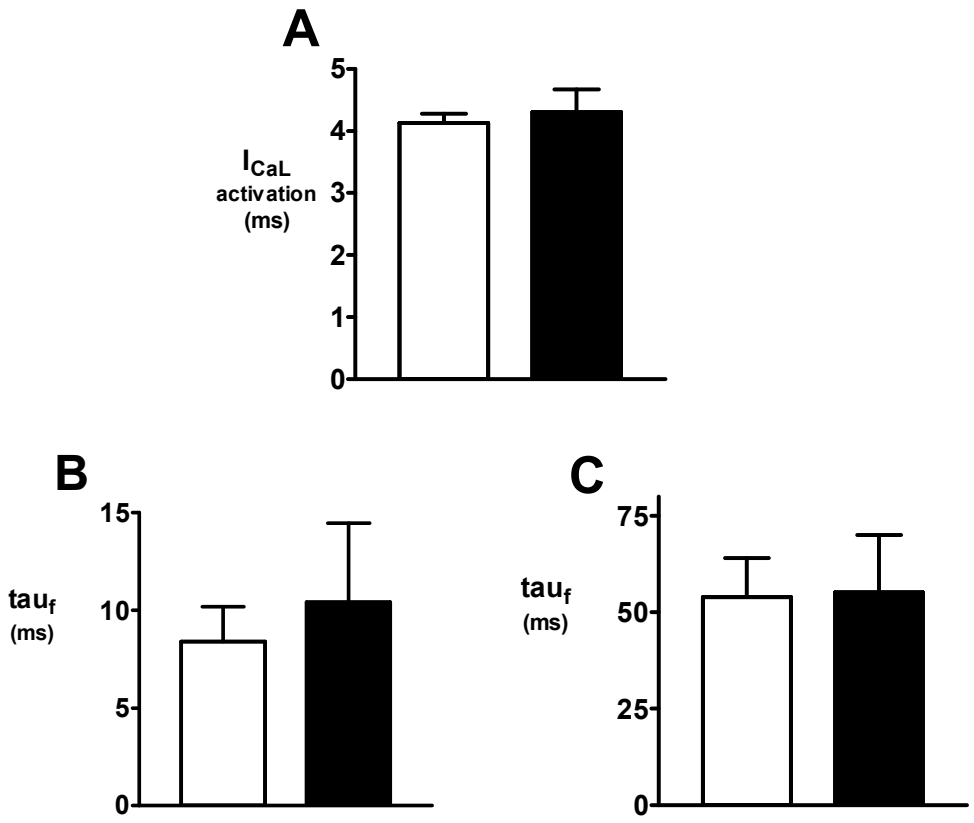


Figure 3.17 The effect of acute superfusion of ISO at 0.05 μM on activation and inactivation time constants of I_{CaL}. □ = control ■ = ISO 0.05 μM ISO (n=4 cells from 4 patients, paired data, mean± s.e.m.).

A The lack of effect of ISO 0.05 μM on the activation time of I_{CaL} (ms)

B The lack of effect of ISO 0.05 μM on the rapid (tau_f) time constant of inactivation of I_{CaL} (ms).

C The lack of effect of ISO 0.05 μM on the slow (tau_s) time constant of inactivation of I_{CaL} (ms).

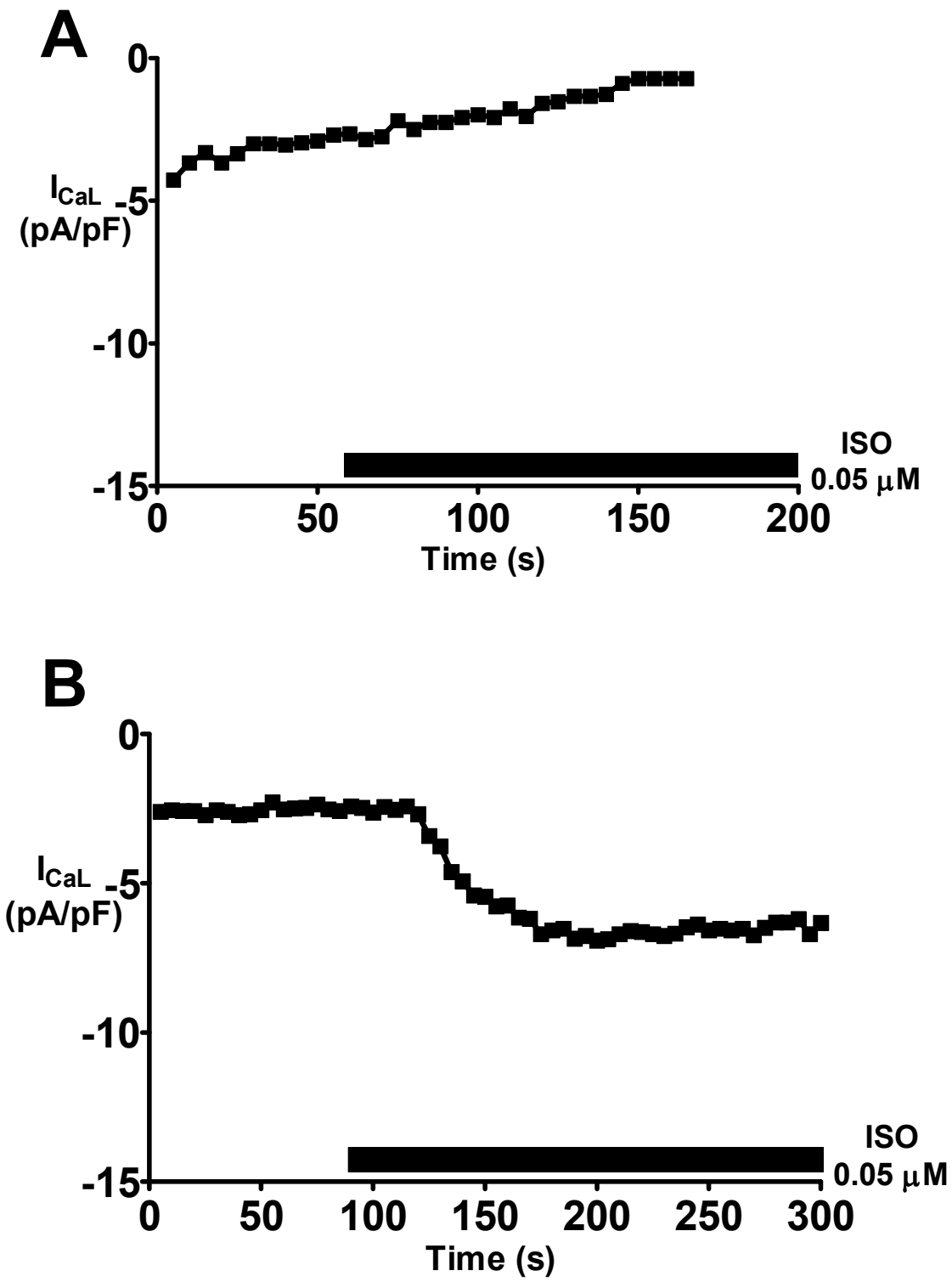


Figure 3.18 Examples of the type 0 and type 1 time course of effect of ISO on I_{CaL}.
A Type 0 effect; perpetual rundown from an initial peak I_{CaL} until cell death following superfusion with ISO.These cells were excluded from further analysis.
B Type 1 effect; stable I_{CaL} under both control conditions and following exposure to ISO until experiment electively terminated.

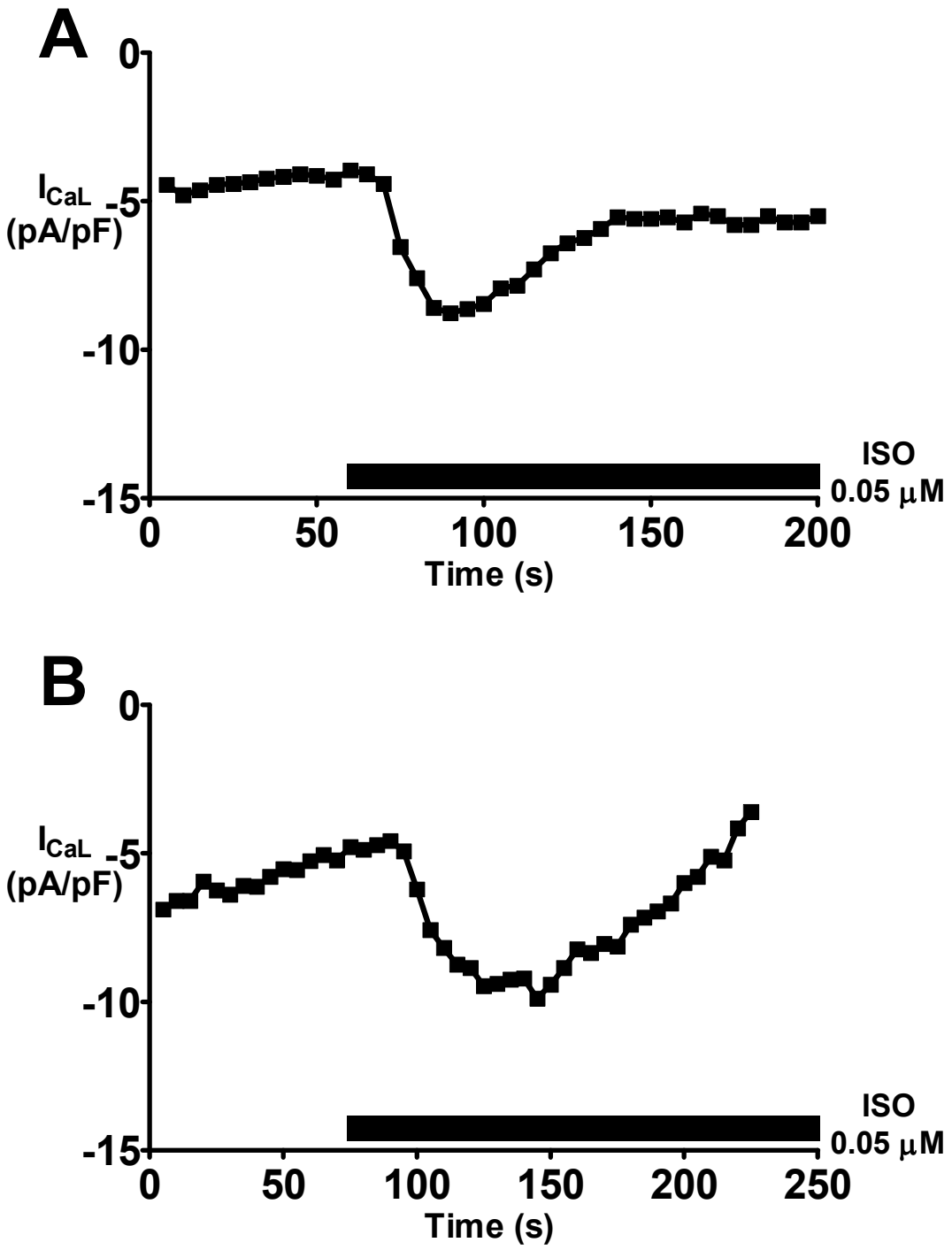


Figure 3.19 Examples of the type 2 and type 3 time course of effect of ISO on I_{CaL}.
A Type 2 effect; stable I_{CaL} under control conditions prior to superfusion with ISO, however the adrenergic effect was an initial rise in peak I_{CaL} follwed by rundown which stabilised at a plateau greater than under control conditions.
B Type 3 effect; following addition of ISO to the superfusate, an initial rise in peak I_{CaL} was followed by irreversible rundown of I_{CaL} until cell death.

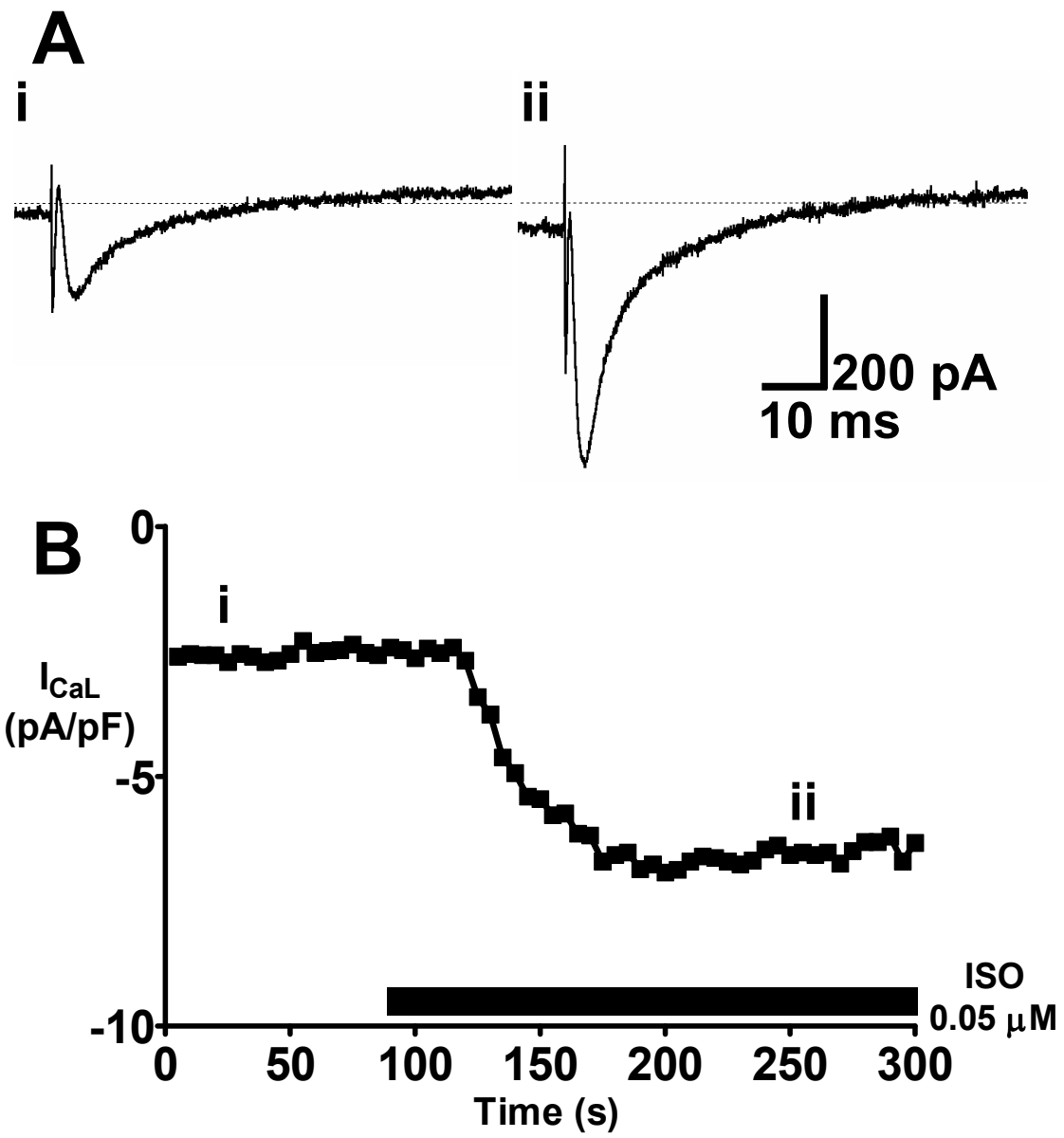


Figure 3.20 Effect of acute superfusion with 0.05 μM isoproterenol on a human atrial myocyte.
A Examples of the raw inward current traces recorded under control conditions (i) and (ii) during acute superfusion with 0.05 μM isoproterenol.
B Representative example of the time course of the adrenergic effect of ISO (0.05 μM) on peak I_{CaL}. Timing of the addition of ISO to the superfusate is indicated by the solid black bar.

3.5.17 Repetitive applications of ISO

Initial experimental protocols were of a stepwise increase in the concentration of ISO in the superfusate following the adrenergic effect reaching a steady state. However, this stepwise effect of ISO was achieved in few cells (Figure 3.24). The estimation of the magnitude of the effect of second and subsequent applications of ISO were hampered by failure of initial plateau formation (type 3 responses), the initial rundown prior to plateau formation negating the impact of further adrenergic effects with increasing concentrations of ISO (type 2 response) See Figure 3.24B.

3.5.18 Impact of the method of seal formation on the adrenergic effect of 0.05 μM ISO on I_{CaL}

Mode of electrical access, (NR v NP), was not associated with any specific 'type' of response of human atrial myocyte I_{CaL} following acute superfusion with ISO in concentrations ranging from 10^{-10} to 10^{-6} M. There was no significant difference (NR v NP) in the magnitude of the adrenergic effect of 0.05 μM ISO on peak I_{CaL} (NR 176.1± 24.8% v. NP 246± 24.8% increase in peak I_{CaL}, n= 22 cells from 15 patients and n= 65 cells from 21 patients respectively).

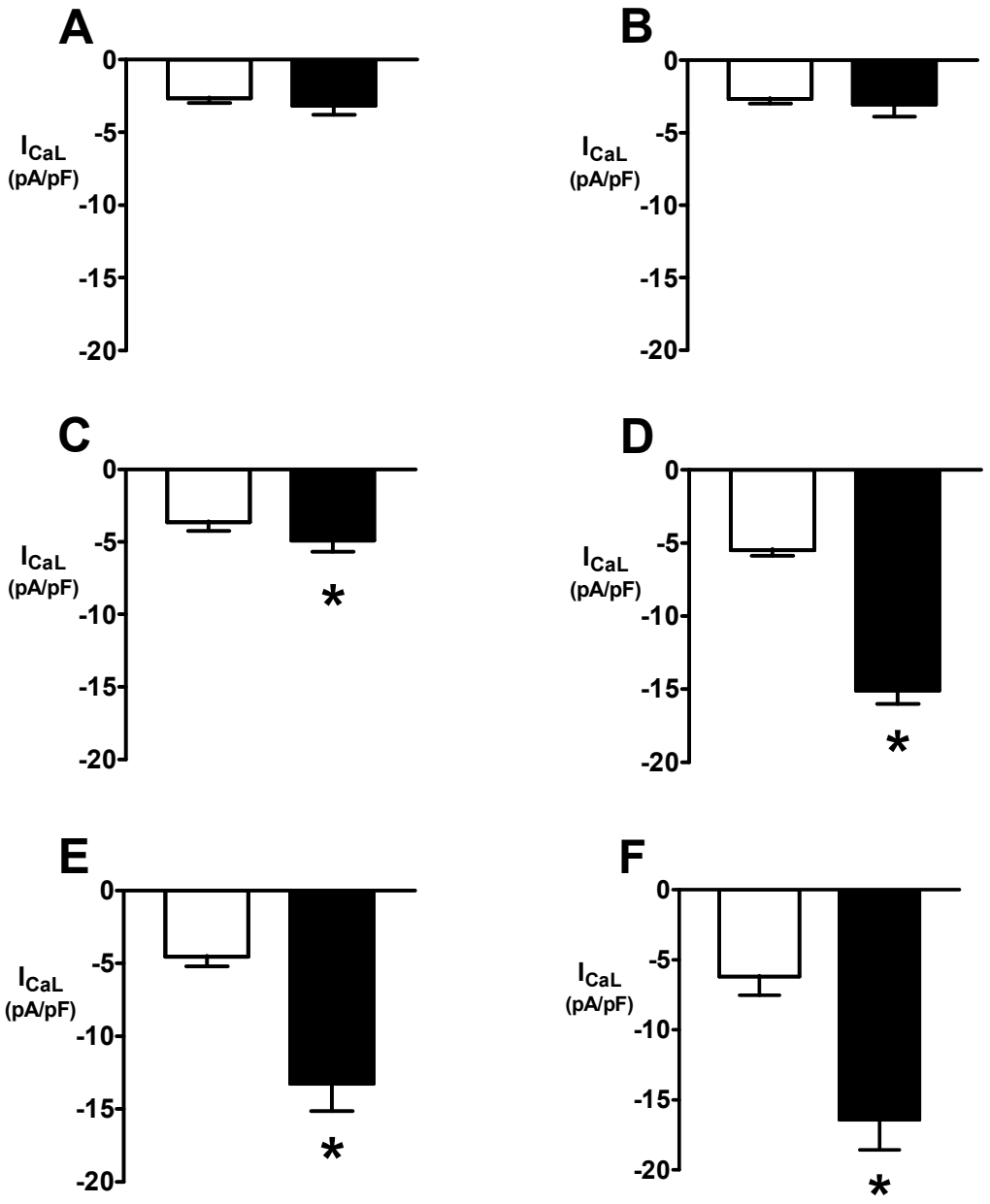


Figure 3.21 The significant adrenergic effect of acute superfusion with increasing concentrations of ISO on peak I_{CaL}. □ = control ■ = ISO (varying concentrations as indicated) paired data, values are mean± s.e.m.
A 0.0001 µM ISO (n= 4 cells from 3 patients)
B 0.001 µM ISO (n= 4 cells from 3 patients)
C 0.01 µM ISO (n= 5 cells from 4 patients, $p< 0.05$.)
D 0.05 µM ISO (n= 87 cells from 36 patients, $p< 0.05$.)
E 0.1 µM ISO (n= 20 cells from 13 patients, $p< 0.05$.)
F 1.0 µM ISO (n= 17 cells from 10 patients, $p< 0.05$.)

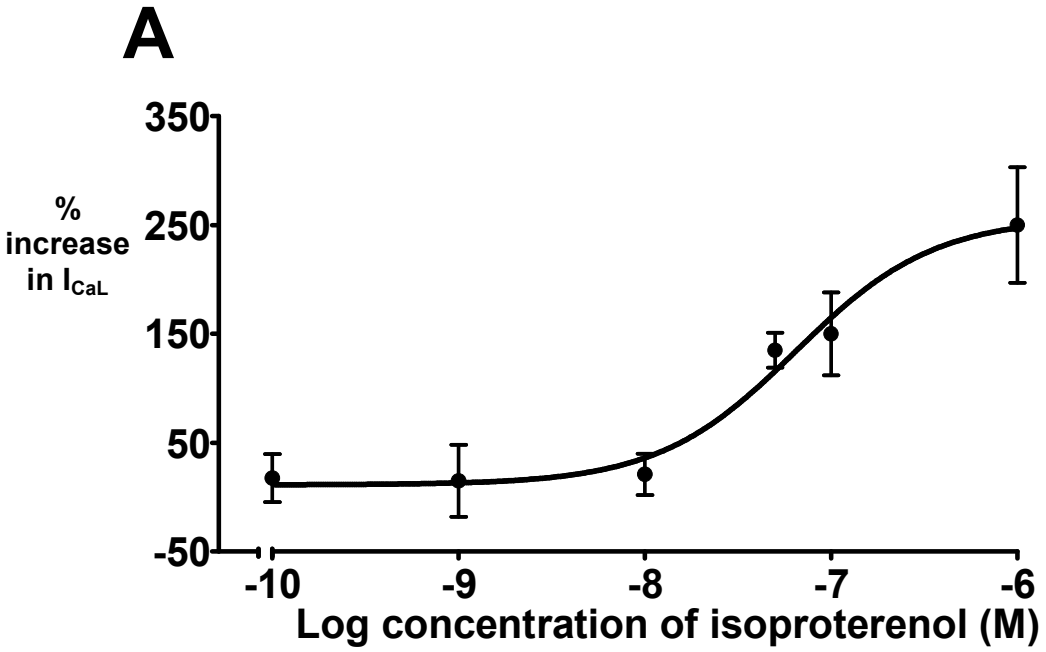


Figure 3.22 Concentration: response curve of the effect of Isoproterenol on I_{CaL}. Data from which an unrestrained sigmoidal plot using the Hill equation calculated the EC_{50} to be 0.06± 0.014 μM ISO, the minimal and maximal effects of ISO were estimated to be 16.5± 21% and 199.3± 43% increases in I_{CaL} compared to baseline respectively (n= 4- 46 cells from 3- 22 patients).

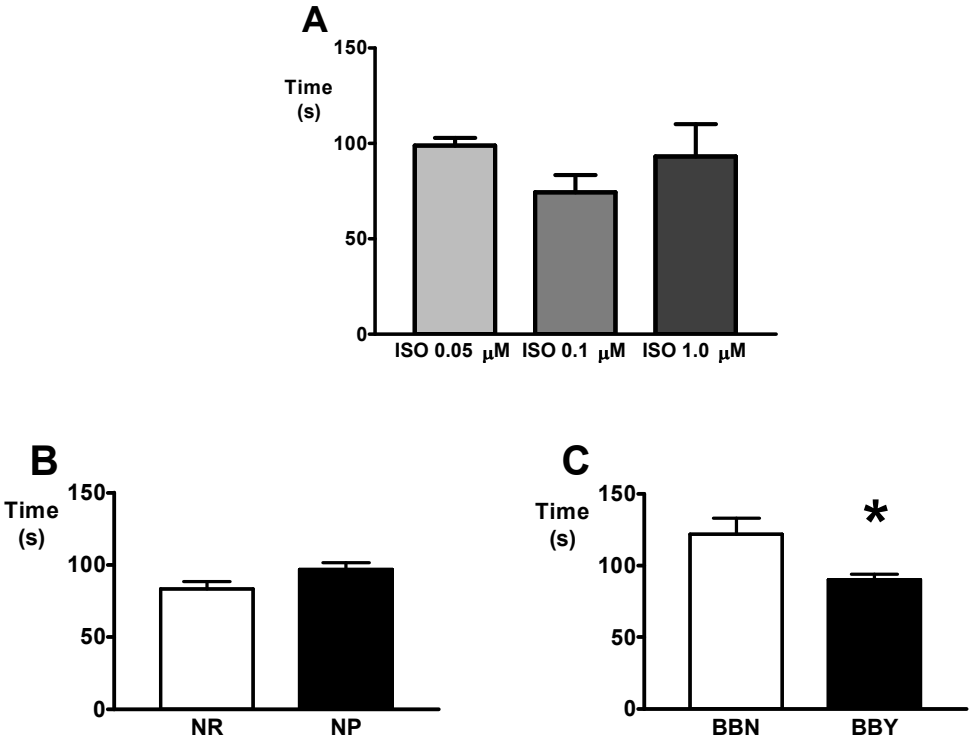


Figure 3.23 Analysis of time to maximal adrenergic effect of ISO on peak I_{CaL}.
A The mean time to maximal effect of differing concentrations of ISO on peak I_{CaL} all values are paired data mean± s.e.m. = 0.05 μM ISO (n= 87 cells from 36 patients) = 0.1 μM ISO (n= 17 cells from 12 patients) = 1.0 μM ISO (n= 11 cells from 7 patients)
B The lack of impact of method of electrical seal on the time to peak ISO effect on I_{CaL} (nystatin perforated patch (NR) n= 92 cells from 38 patients v nystatin ruptured patch (NR) n= 21 cells from 13 patients, mean± s.e.m.)
C Chronic pre-operative β-B therapy results in an expedited peak response to ISO (mean± s.e.m.). n= 97 cells from 37 patients receiving chronic pre-operative β-B therapy (BBY). n= 16 cells from 7 patients not receiving chronic pre-operative β-B therapy (BBN).

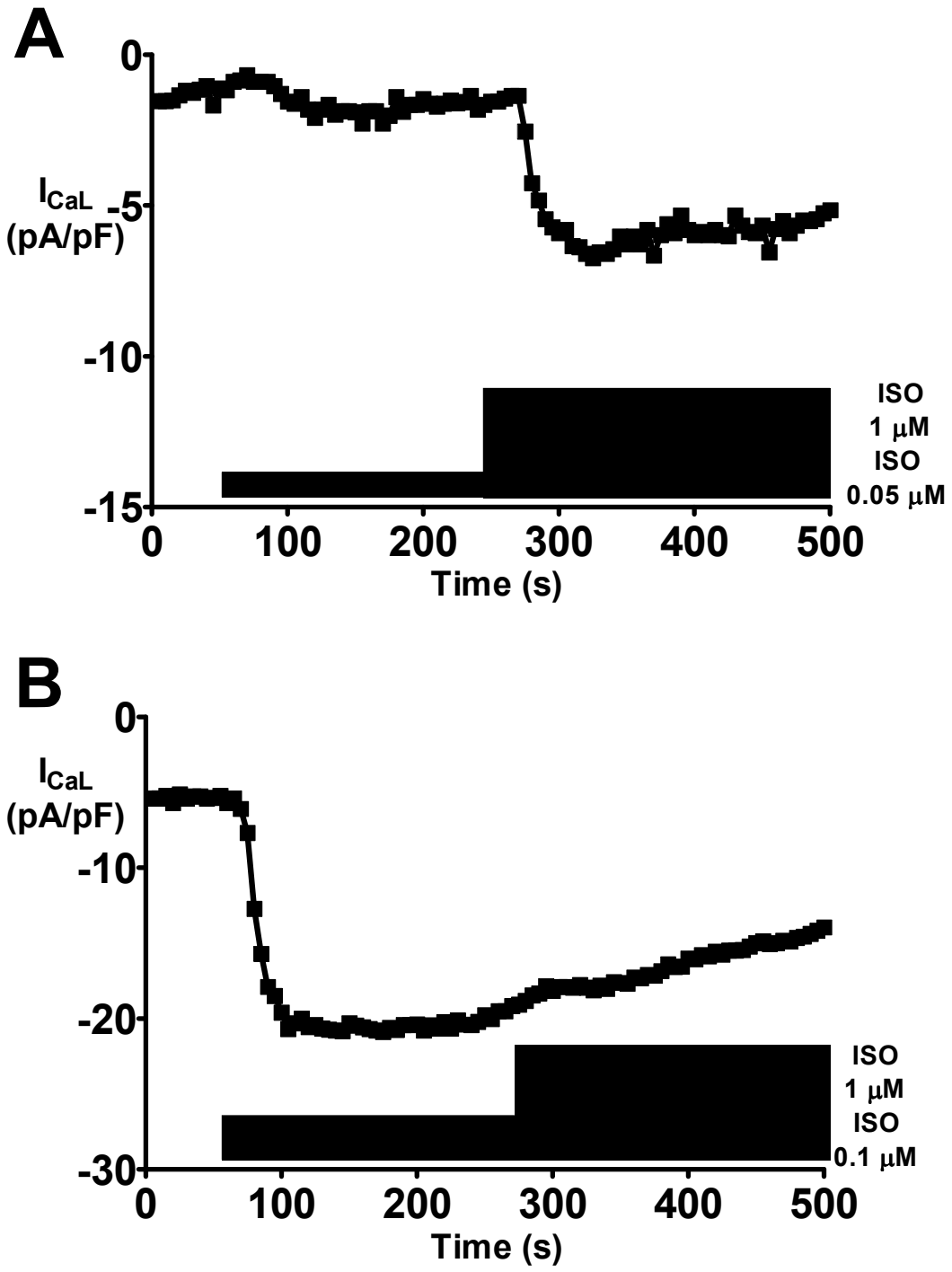


Figure 3.24 Differing cellular responses to repetitive applications of increasing concentrations of Isoproterenol.
A Repetitive application of ISO in which subsequent addition of an increased concentration of ISO to the superfusate resulted in a stable increase in the magnitude of peak I_{CaL}
B Increased concentration of ISO did not reverse the onset of I_{CaL} rundown.

3.5.19 Impact of patients' clinical characteristics on the adrenergic effect of 0.05 μM ISO on I_{CaL}

There were no significant differences observed in the magnitude of the mean adrenergic effect of 0.05 μM ISO on peak I_{CaL} associated with any recorded patient clinical characteristics or pre-operative drug therapy, other than the presence of pre-operative hypertension. The mean adrenergic response of peak I_{CaL} to 0.05 μM ISO was significantly greater in myocytes isolated from patients suffering from hypertension pre-operatively as compared to myocytes from patients free from hypertension. (Mean adrenergic increase 250.7± 25.3% n= 46 cells from 19 hypertensive patients v. 173.5± 22.2% n= 38 cells from 15 normotensive patients).

3.5.19.11 Adrenergic response of peak I_{CaL} to ISO 0.05 μMol is not predictive of post operative Atrial Fibrillation (AF_{pcs})

The mean adrenergic increase in peak I_{CaL} from myocytes isolated from those patients who went on to develop post-operative Atrial Fibrillation (AF_{pcs}) was not significantly different from the mean adrenergic increase in peak I_{CaL} of myocytes from patients who remained in sinus rhythm post-operatively (SR_{pcs}). (Mean adrenergic increase (AF_{pcs}) 193.7± 32.7% n= 28 cells from 12 patients v. (SR_{pcs}) 229.2± 20.1% n= 58 cells from 23 patients).

3.5.20 The lack of effect of Endothelin-1 alone on peak I_{CaL}

Endothelin-1 alone at the maximal concentration tested of 10 nM had no effect on peak I_{CaL} (control -4.8± 0.5 pA/pF v. ET-1 -4.5± 0.7 pA/pF, n= 5 cells from 4 patients, paired data, $p< 0.05$ Figure 3.25). An example of this lack of effect of ET-1 at 10 nM on I_{CaL} is shown in Figure 3.25A and is confirmed by mean data (Figure 3.25B).

3.5.21 The lack of effect of Sarafotoxin-S6c alone on peak I_{CaL}

Sarafotoxin-S6c, a selective ET-$_B$ receptor agonist, at 10 nM had no effect on I_{CaL} (control -3.9± 1.1 pA/pF v. S6c -3.3± 0.9 pA/pF, n= 5 cells from 3 patients, paired data, p< 0.05 Figure 3.26). An example of this lack of effect of S-6c on I_{CaL} is shown in Figure 3.26A and is confirmed by mean data (Figure 3.26B).

3.5.22 The effect of Endothelin-1 on adrenergically pre-stimulated peak I_{CaL}

Peak I_{CaL} was pre-stimulated with ISO at a concentration of 0.05 μM which approximated to the EC_{50} of the adrenergic stimulatory effect of ISO on peak I_{CaL}. Once it was apparent in real time that the adrenergic effect had reached a plateau, Endothelin-1, in varying concentrations, was added to the superfusate. Endothelin-1, at the maximal concentration evaluated of 10 nM reversed the adrenergic effect of 0.05 μM ISO on peak I_{CaL}, as can be seen in Figure 3.27A (control -4.9± 1.3 pA/pF v. ISO -16.8± 3.4 pA/pF v. ISO and ET-1 co-application -8.2± 2.1 pA/pF n= 14 cells from 12 patients, paired data, p= 0.0005 Figure 3.27B). The anti-adrenergic effect was calculated by first measuring the magnitude of the adrenergic effect of ISO on I_{CaL} in absolute terms and then dividing the magnitude of the effect of ET-1 in pA/pF permitting the expression of the anti-adrenergic effect of ET-1 as a percentage of the initial adrenergic effect (See Figure 3.27).

3.5.23 Partial reversibility of the anti-adrenergic effect of ET-1 on peak I_{CaL} due to washout

The anti-adrenergic effect was partially reversible with washout in the majority of cells tested (Figure 3.28). In 10 of 16 cells in which the experiment continued into a washout period, the effect of ET-1 was partially reversible. During washout of all

concentrations of ET-1 ≤1 nM partial reversibility was more frequent than not. In 5 cells it was not possible to assess reversibility due to premature termination of the experiment due to cell death or electrical instability. No correlation between preoperative drug therapy or comorbidity could be made as the numbers in each group preclude further investigation.

3.5.24 The concentration: response relationship of the anti-adrenergic effect of ET-1 on peak I_{CaL}

The magnitude of the anti-adrenergic effect of decreasing concentrations of ET-1 was assessed in order to determine if there was a relationship between the concentration of ET-1 and the magnitude of the anti-adrenergic effect on peak I_{CaL}. Endothelin-1 had a significant anti-adrenergic effect on peak I_{CaL} in all concentrations tested from 0.01 nM to 10 nM (See Figure 3.29). The beginning of a concentration response effect can be determined as the magnitude of the anti-adrenergic effect decreased with decreasing concentrations of ET-1 in the superfusate.

3.5.25 The concentration response relationship of the time course of the anti-adrenergic effect of ET-1 on peak I_{CaL}

The time taken for ET-1 to have an anti-adrenergic effect appeared to be concentration dependent. At high concentration, ET-1 at 10 nM completely reversed the adrenergic effect of ISO at 0.05 μM in 140± 9 seconds which was significantly earlier than lower concentrations (n= 4-14 cells from 2-12 patients P= 0.0004. Figure 3.30).

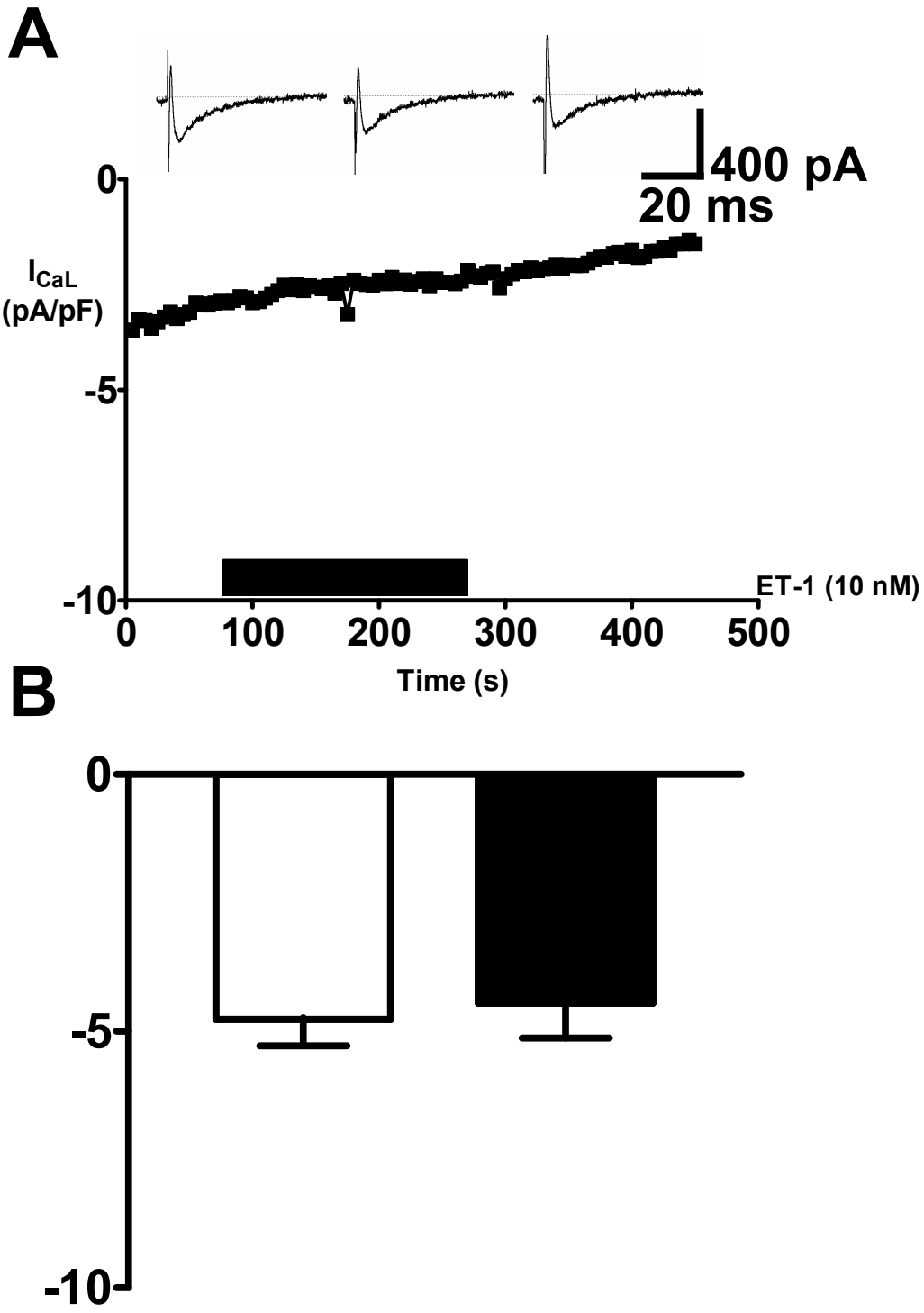


Figure 3.25 A) Time course of 10 nM ET-1 effect, inset are examples of raw current tracings obtained during each phase of the experiment. B) Histograms of mean data of 10 nM ET-1 effect on peak I_{CaL}. □ = control ■ = ET-1 10 nM (n= 5 cells from 4 patients, paired data mean ± s.e.m., p= ns).

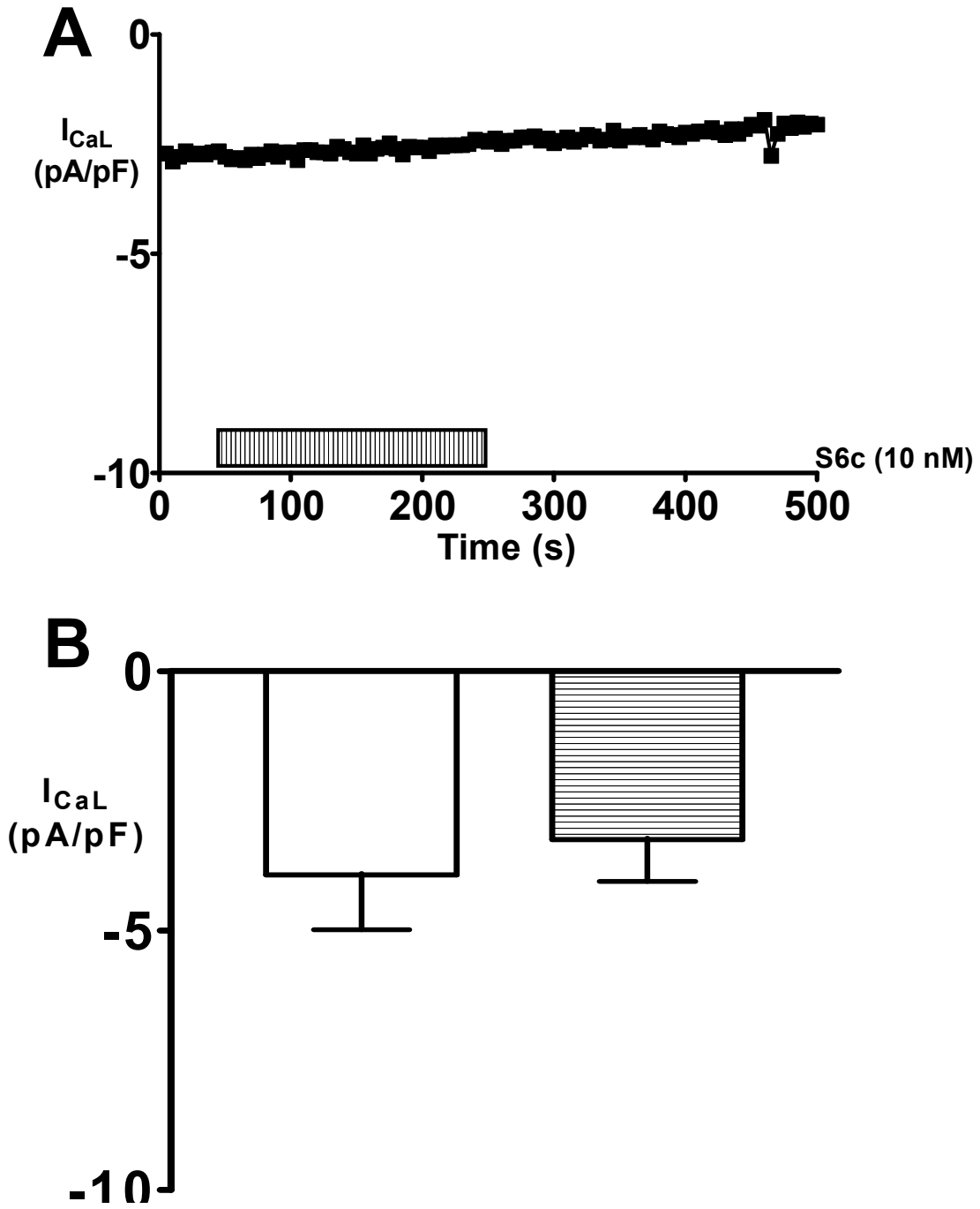


Figure 3.26 A) Time course of lack of effect of 10 nM S6c on I_{CaL}. B) Histograms of mean data of 10 nM S6c effect on peak I_{CaL}. □ = control ▒ = S6c 10 nM (n= 5 cells from 3 patients, paired data mean ± s.e.m., p= ns).

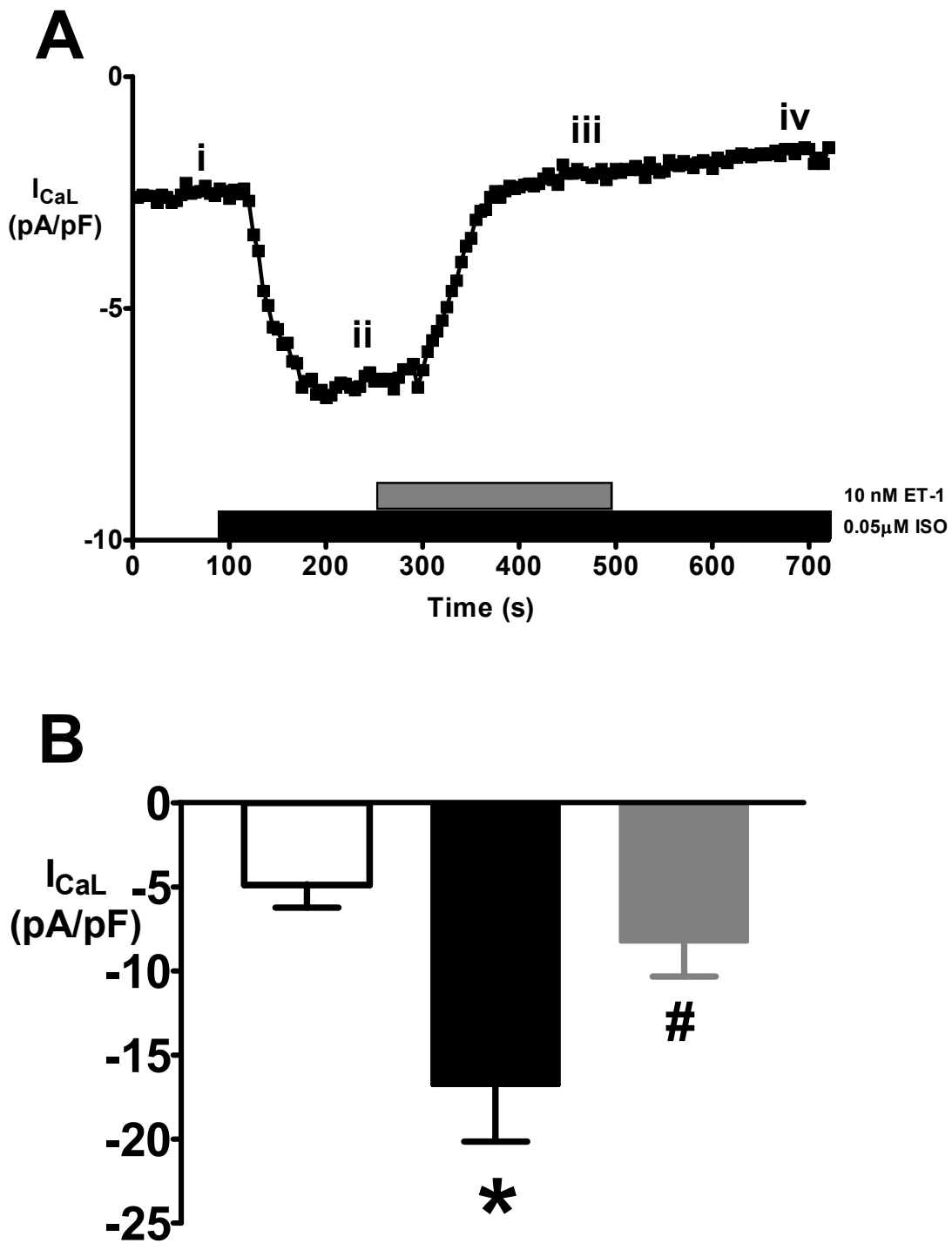


Figure 3.27 A) Time course of the anti-adrenergic effect of 10 nM ET-1 on peak I_{CaL}. B) Histogram of mean data of the anti-adrenergic effect of 10 nM ET-1 on peak I_{CaL}. □ = control ■ = ISO 0.05 μM ■ = ET-1 10 nM (n= 14 cells from 12 patients, paired data, mean ± s.e.m., * P= 0.0005, # P= 0.0002).

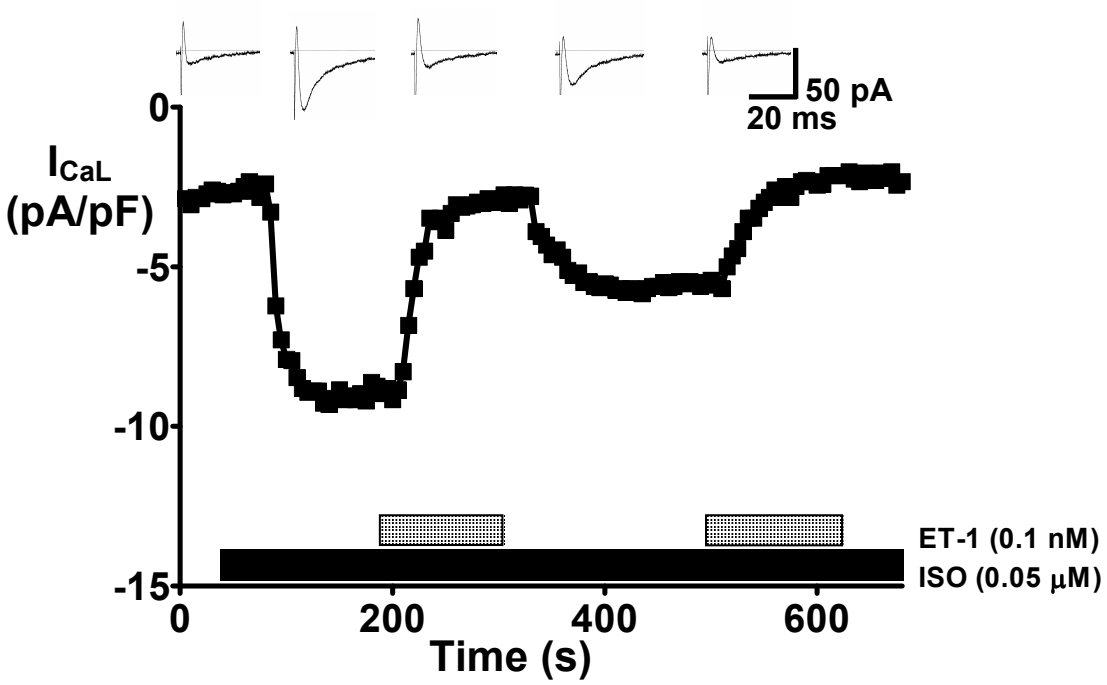


Figure 3.28 Example of the partial reversibility of the anti-adrenergic effect of 0.1 nM ET-1 on peak I_{CaL} with washout.

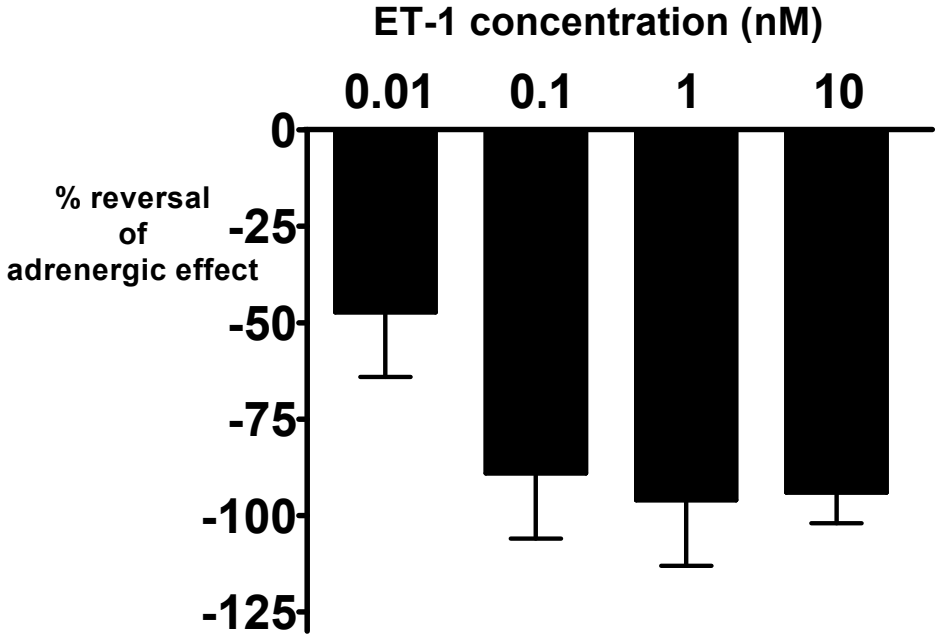


Figure 3.29 The concentration response relationship of the magnitude of the anti-adrenergic effect of ET-1 (nM) on peak I_{CaL}.
10 nM ET-1 n = 14 cells from 12 patients P= 0.0002
1 nM ET-1 n = 4 cells from 2 patients P= 0.017
0.1 nM ET-1 n = 5 cells from 3 patients P= 0.013
0.01 nM ET-1 n = 5 cells from 5 patients P= 0.036

3.5.26 Partial reversibility due to the blockade of ET-$_A$ receptors with FR139317 (FRA)

Using the ET-$_A$ antagonist FRA the effect of 10 nM ET-1 was partially reversible in 6 of 7 seven cells (Figure 3.31).

3.5.27 The blockade of ET-$_A$ receptors with FR139317 (FRA) prevents the antiadrenergic effect of 10 nM ET-1 on adrenergically pre-stimulated peak I_{CaL}

Superfusion of the myocyte with the ET-$_A$ antagonist FRA following exposure to 0.05 μM ISO prevented the anti-adrenergic effect of 10 nM ET-1 on peak I_{CaL} (Figure 3.32).

3.5.28 The lack of effect of Sarafatoxin-S6c on adrenergically pre-stimulated peak I_{CaL}

The selective ET-$_B$ receptor agonist Sarafotoxin-S6c at 10 nM had no effect on I_{CaL} which had been adrenergically pre-stimulated with 0.05μM ISO (control 4.7± 0.5 pA/pF v. ISO -19.2± 2.7 pA/pF v. ISO and S6c co-application 18.0± 3.4 pA/pF, paired data n=9 cells from 4 patients). An example of this lack of effect of S-6c on I_{CaL} is shown in Figure 3.33 and is confirmed by mean data (Figure 3.34). The presence of 10 nM S6-c in the superfusate had no effect on the ability of 10 nM ET-1 to reverse the adrenergic effect of 0.05μM ISO on peak I_{CaL} (Figure 3.43B).

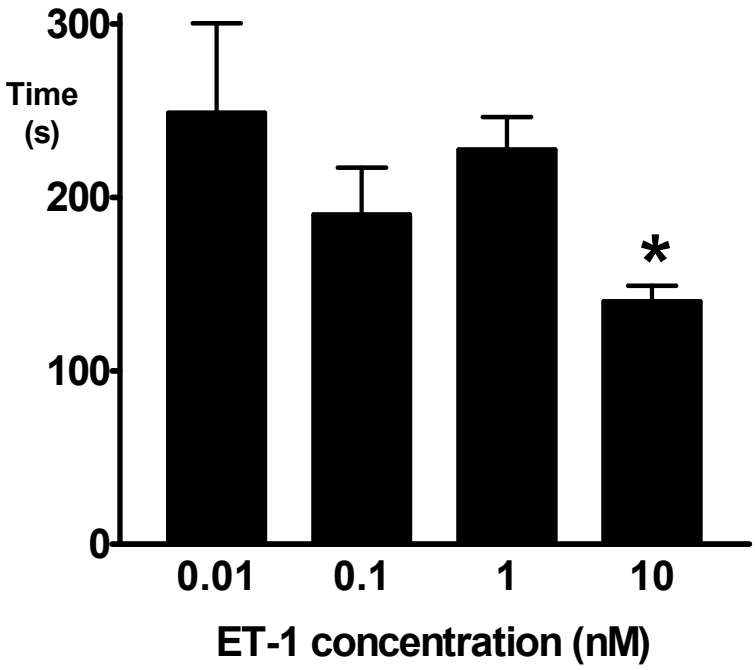


Figure 3.30 The concentration response effect of the time to peak anti-adrenergic effect of ET-1 on peak I_{CaL}. (n= 4-14 cells from 2-12 patients. * P= 0.0004)

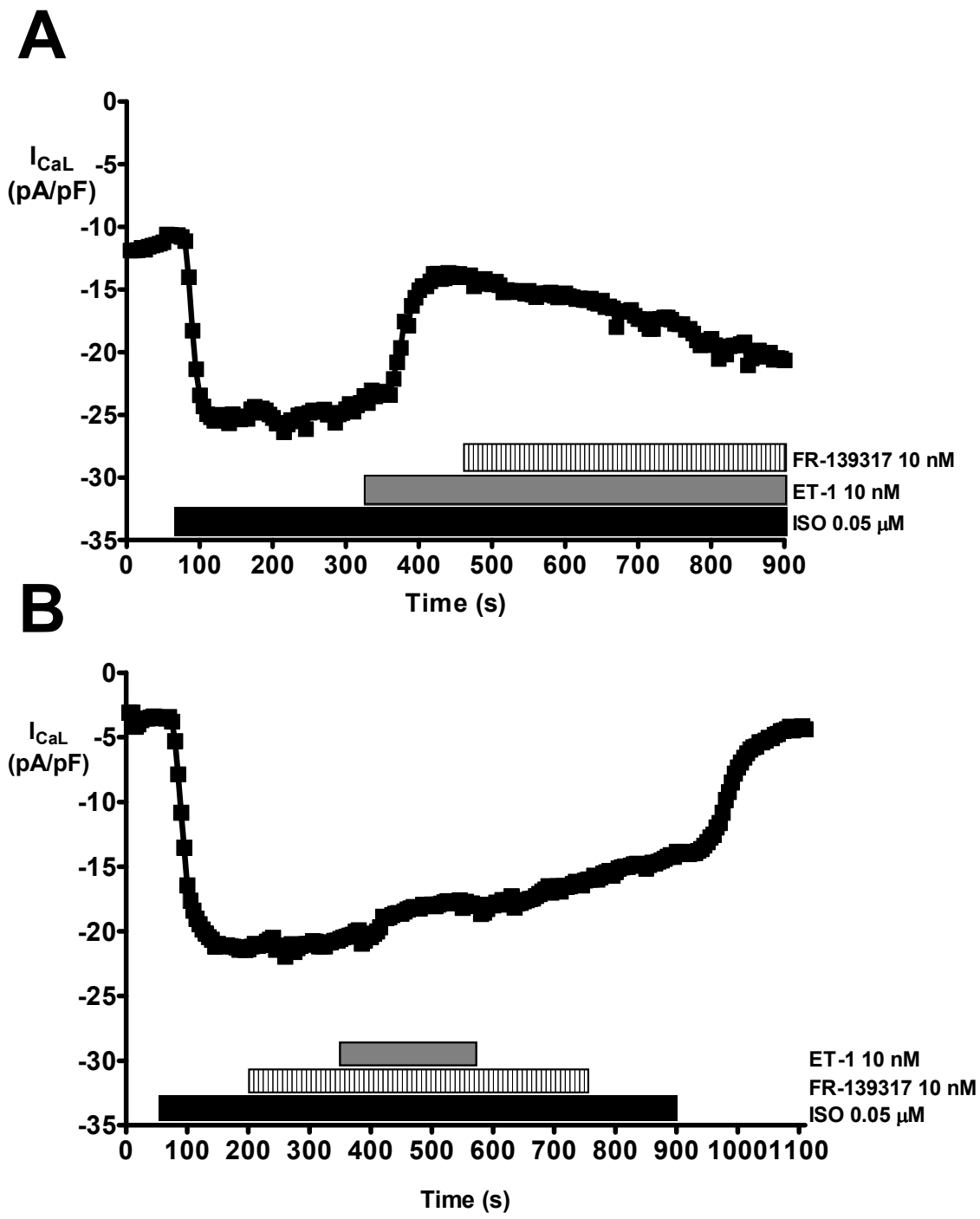


Figure 3.31 A) The specific ET-$_A$ receptor antagonist FR-139317 at 10 nM partially reverses the anti-adrenergic effect of 10 nM ET-1 on peak I_{CaL}. B) The specific ET-$_A$ receptor antagonist FR-139317 at 10 nM prevents the anti-adrenergic effect of 10 nM ET-1 on peak I_{CaL}.

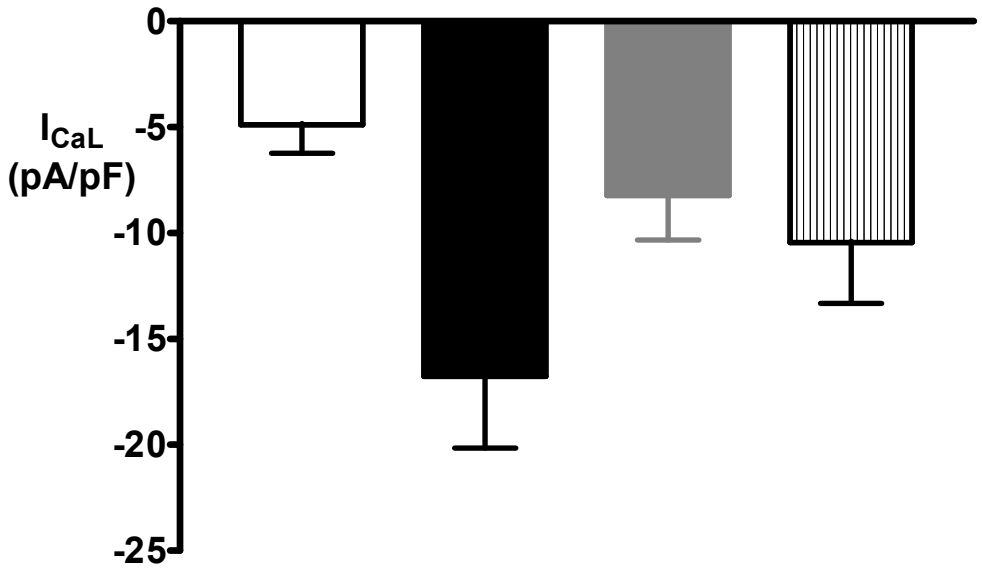


Figure 3.32 The mean effect of 10 nM FR139317 to partially reverse the anti-adrenergic effect of 10 nM ET-1 on peak I_{CaL}. □ = control ■ = ISO 0.05 μM ▒ = ET-1 10 nM ▥ = 10 nM FRA (n = 6 cells from 4 patients mean ± s.e.m. p= ns).

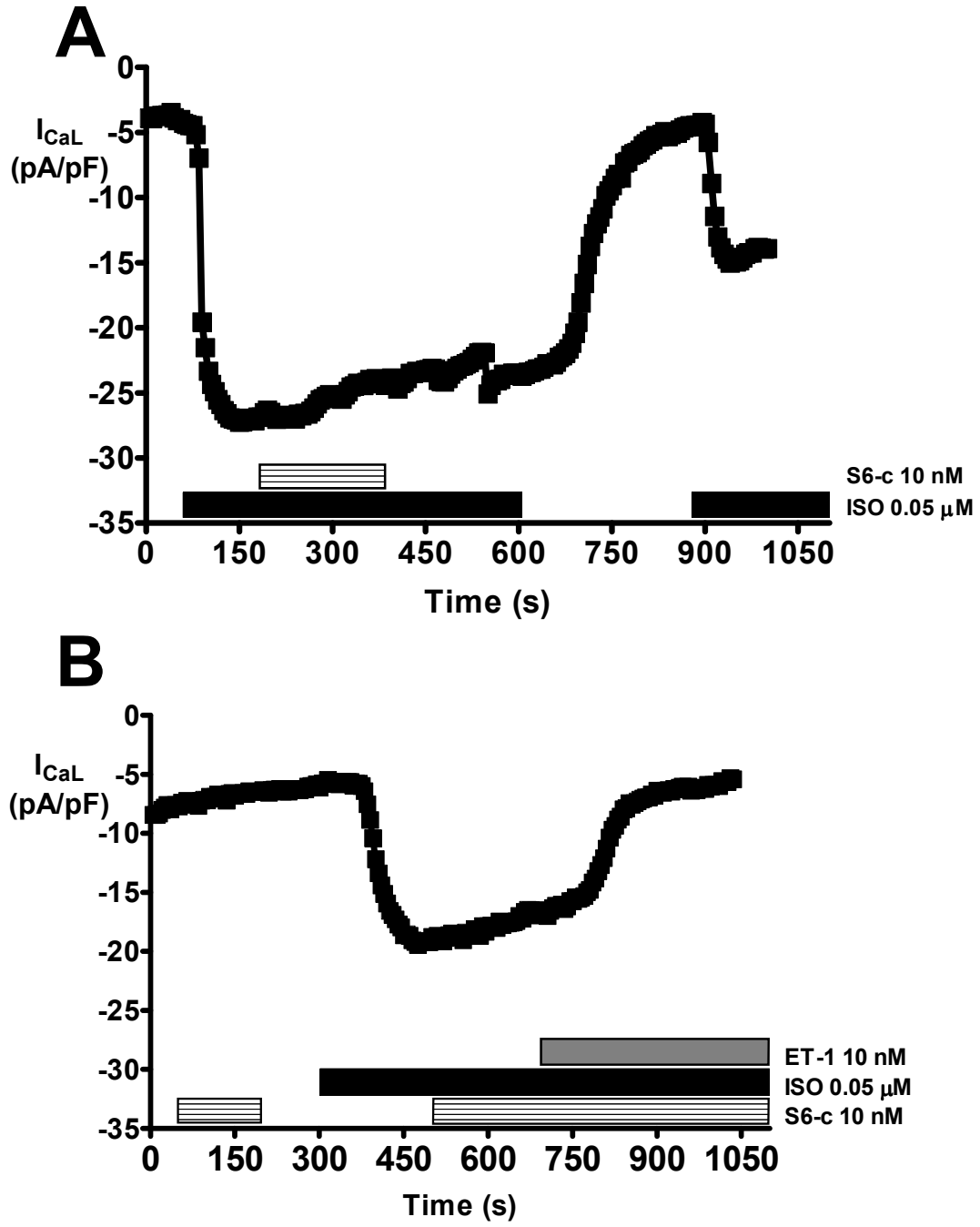


Figure 3.33 A) The lack of effect of Sarafotoxin S6-c alone on prestimulated peak I_{CaL}. B) The absence of effect of Sarafotoxin S6-c on the anti-adrenergic effect of 10 nM ET-1 on peak I_{CaL}.

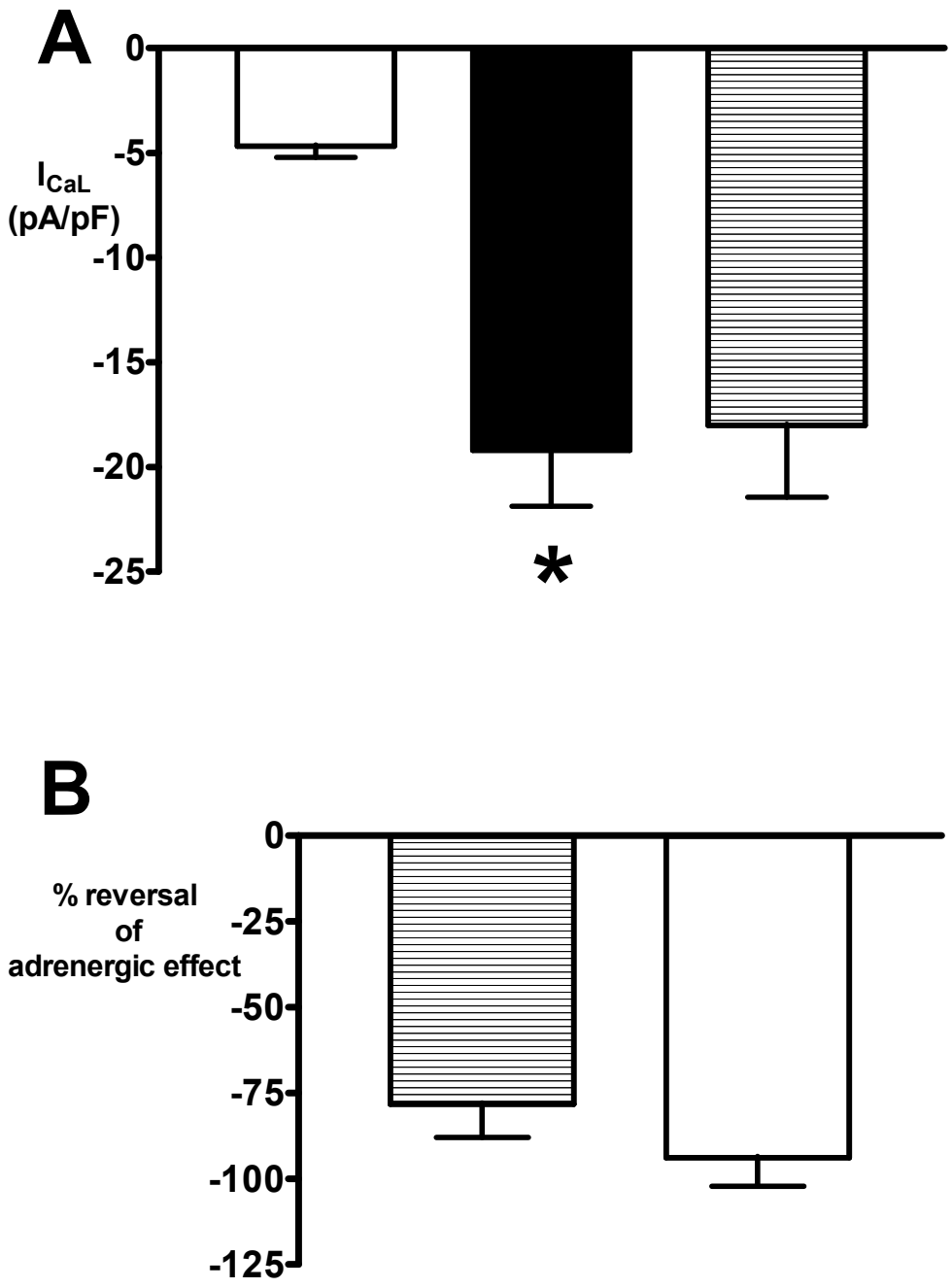


Figure 3.34 A) Mean lack of effect of sarafotoxin S6-c on adrenergically prestimulated peak I_{CaL} . □ = control ■ = ISO 0.05 μM ▒ = S6c 10 nM (n= 9 cells from 4 patients mean ± s.e.m.) B) The impact of sarafotoxin S6-c on the anti-adrenergic effect of 10 nM ET-1 on peak I_{CaL} (ET-1 alone n= 14 cells from 12 patients, ET-1 following exposure to S6-c n= 3 cells from 1 patient mean ± s.e.m. p= ns)

3.6 Discussion

3.6.1 Methodological considerations

These results demonstrate that I_{CaL} can be reliably and reproducibly recorded from human atrial myocytes using the nystatin-perforated whole-cell patch clamp technique at physiological temperature. This nystatin perforated method had no significant effect on activation nor inactivation kinetics, the current: voltage relationship nor peak inward calcium current while avoiding the irreversible current rundown in the vast majority of cells under control conditions[79;161;163;223]. Additionally these results indicate that the method of electrical access, patient gender and chronic preoperative drug therapy had no effect on human atrial I_{CaL} under control conditions. In contrast, in cells isolated from patients suffering from moderate and severe LVSD, as defined by trans-thoracic echocardiography (TTE), there was a significantly greater peak I_{CaL} observed with no significant alteration in the I-V relationship.

The clinical characteristics of the patients from whom these atrial myocytes were isolated are similar to those reported in recent surgical series[283] and on the UK central cardiac audit database (www.ccad.org.uk/ccadweb.nsf). Due to the small sample size of patients in the present study it was not possible to detect any significant associations between clinical characteristics and post-operative AF, however this would appear to be a type II error as pooled work from our laboratory has recently been published reporting the presence of these associations[284].

An association between pre-operative ACE-I therapy and greater R_s has not, as far as the author is aware, been previously reported. Angiotensin II has been implicated in stretch induced fibrosis, reversible increases in cellular expression of connexin 43 and other membrane bound proteins, however, these changes were

not associated with alterations in resting membrane potential or action potential upstroke[285;286]. While this possible effect of chronic pre-operative ACE-I therapy on human atrial myocyte plasmalemmae cannot be entirely discounted it is likely to be a spurious result representing a type I error. This interpretation is supported by the publication of pooled data from our laboratory which did not report any significant association between ACE-I therapy and greater R_s[284].

Successful perforated patch recordings were more likely with two periods of enzymatic incubation during the myocyte isolation process and also in cells of greater capacitance. Nystatin-induced pores in the lipid bilayer permit electrical access and in comparison with ruptured patch where the pipette is in direct contact with the intracellular milieu it is intuitive that R_s should be greater in NP cf. NR recordings. Exactly how nystatin pores, cell size and pre-operative ACE-I therapy interact at the plasmalemmae remains a subject for further research. The present study confirms that the stability of I_{CaL} recordings can be maintained far beyond that which was previously possible with ruptured patch recordings[202;220;221].

The majority of previous publications describing I_{CaL} used ruptured patch clamp experiments at room temperature [114;155;163;225;281;282;287;288] and all report a bell shaped I-V relationship with inward current being detected in the range of >-40 mV to <+60 mV, peaking at +10± 10 mV. This 'peak' I_{CaL}, measured either at 0 mV or at +10 mV in these experiments was in the range of 1.2 pA/pF [225] to 3.2 pA/pF [287]. Early pioneering work in this area was hampered by I_{CaL} being detected in fewer than half of cells studied and often required amplification with dihydropyridine agonists (Bay K 8644) in order to become recordable[155].

In contrast when physiological temperature conditions were employed, the current voltage relationship of I_{CaL} was bell shaped with peak current consistently at +10

mV[79;161;163;223]. One report specifically examined the physiological effect of temperature on I_{CaL}[161] demonstrating no shift in the IV relationship but an increase in peak I_{CaL} from 2 pA/pF at room temperature increasing to 6 pA/pF from a holding potential of -40 mV at physiological temperature. When the effect of raising the temperature of the cells during the experiment was tested directly it was demonstrated that I_{CaL} had similar I: V relationship but that greater current passed at all voltages and that peak current was approximately doubled with more rapid activation and inactivation[161]. Further corroboration has arisen from subsequent work reporting that peak I_{CaL} was in the range of 4.8 to 6.8 pA/pF occurring at +10 mV under control conditions of 37^0C[79;144;161;163;223].

3.6.2 Interaction of patients' clinical characteristics and human atrial isolated myocyte I_{CaL}

Previous publications have sought to determine whether chronic pre-operative drug therapy alters human atrial myocyte electrophysiology. This was suggested by reports indicating altered contractile responses and arrhythmogenicity in cells from patients who had received chronic preoperative beta-adrenoceptor (BB) antagonists [152;289-291]. The data presented are in accordance with previous work, including work from our own laboratory, demonstrating that pre-operative therapy with BB has no significant effect on human atrial I_{CaL} under control conditions[144;223;281;287].

Initial assessments of the impact of chronic therapy with calcium channel blockers (CCB) pre-operatively reported no effect on the I-V relationship of I_{CaL} but reported an association with reduced peak I_{CaL} under control conditions [155;282]. No such association was apparent in this study. The association of reduced peak I_{CaL} and chronic CCB therapy has not been corroborated since the initial two reports

[144;223;281;287]. Possible explanations are methodological differences, a type I error as both reports contain data from few patients and limited cell sample sizes and early experimental difficulties in recording I_{CaL}[155].

Treatment with ACE-Inhibitor drugs, while not considered anti-arrhythmic therapy in the traditional sense have recently been associated with preventive[292-295] and supportive effects in the successful cardioversion of atrial fibrillation[296;297]. Under control conditions there was no significant difference in peak I_{CaL} between cells from patients taking ACE-I therapy preoperatively and those not. This corroborates previously published data which have not demonstrated a statistically significant electrophysiological effect of preoperative ACE-I therapy, implicating structural or cellular remodelling processes[144;223;281;287].

Gender has been implicated in altered human atrial action potential repolarisation[298]. No gender differences were identified in the measured characteristics of I_{CaL} under control conditions in this study nor in the published literature describing I_{CaL} in vitro[144;223;281;287].

The same group who published the association between chronic preoperative CCB therapy and reduced basal I_{CaL} subsequently described alterations in I_{CaL} comparing ‘normal’ to ‘diseased’ human cardiac cells[114]. Ouadid et al reported that myocytes from patients with severe heart failure requiring cardiac transplantation had significantly greater capacitance and smaller I_{CaL} current density when compared to cells from patients undergoing cardiac surgery for coronary artery disease or mitral stenosis[114]. Although no patients undergoing cardiac transplantation were included in this study, both studies enrolled patients with CHD undergoing cardiac surgery regardless of LVSD or valvular heart disease.

Both studies are in agreement that heart failure, however so defined, was not associated with an alteration in the I-V relationship of I_{CaL}.

In contrast, the present study, under more physiological experimental conditions and with a much greater sample size, has demonstrated that, under control conditions, peak I_{CaL} current density was significantly greater in cells isolated from patients with subjectively moderate/ severe LVSD compared with those with normal/ mild LVSD and that no significant difference in capacitance existed between these two groups. In comparison with Ouadid et al[114], when cells from patients suffering from severe LVSD were considered alone, the association was maintained. However, the subjective presence or absence of LVSD in the present study was associated with similar kinetics, I:V relationship and peak I_{CaL}. More recent data, again recorded at room temperature[299] reported very small peak I_{CaL} (some as low as 0.1 pA/pF) in patients with LVSD. The conclusions presented herein are based on the largest single experimental dataset published to date and are believed to be sufficiently large to permit accurate interpretation and avoidance of type II error.

In order to resolve this issue pooled data from our laboratory submitted for publication with approximately a threefold sample size, (and work from another laboratory[279]) has indicated that atrial myocytes isolated from patients with LVSD, defined objectively by TTE, do have greater capacity and that I_{CaL} has similar kinetics of activation and inactivation, I: V relationship and peak current magnitude regardless of the presence of LVSD after multivariate analysis[300].

3.6.3 Adrenergic effect(s) of acute superfusion with ISO

Isoproterenol, at concentrations ranging from 0.01 to 1 μM, increased the magnitude of the long lasting inward calcium current (I_{CaL}) in a concentration-dependent manner without altering the I:V relationship, activation or inactivation kinetics in human isolated atrial myocytes. In the majority of cells studied, this adrenergic increase in I_{CaL} was stable with a time to maximal effect of approximately 90 seconds. The mode of electrical access did not appear to affect the magnitude of the adrenergic effect, however as indicated in Results chapter 1, perforated patch recordings were associated with a greater duration of stable recordings. Patient senescence and gender did not appear to affect the adrenergic increase in I_{CaL}. Pre-operative drug therapy was not associated with an altered response to acute superfusion with Isoproterenol at the EC_{50} (0.05 μM). A diagnosis of pre-operative hypertension was associated with a significantly larger adrenergic response to 0.05 μM ISO. In contrast, prior MI, the presence of LVSD, regardless of severity, and Aortic Valvular Heart Disease were not associated with altered adrenergic responses. The magnitude of the adrenergic response of I_{CaL} to 0.05 μM was not predictive of the development of post-operative Atrial Fibrillation (AF_{pcs}).

ISO increased the magnitude of calcium current passing into the cell at all voltages recorded in the absence of a detectable change in the current: voltage (IV) relationship. The time to peak current recorded at +10 mV was less than 4 milliseconds in line with one previous report[161]. The concentration: response curve of the effect of ISO on I_{CaL} at physiological temperature using the nystatin perforated patch clamp method has not been previously published. However, our data is similar in comparison with the concentration: response curve of the inotropic effect of ISO in human right atrial preparations[153] reporting an EC_{50}

between 0.01 and 0.1 μM ISO and a maximal effect at 1 μM ISO. The EC_{50} of the stimulatory effect of ISO on cAMP in human right atrial strips was reported to be $10^{-6.23}$ with a maximal effect between 1 and 10 μM ISO[145;146;228;301] resulting in a threefold increase in adenylate cyclase activity[301]. Exposure to ISO resulted in a rapid increase cAMP over 60 seconds but the positive inotropic effect developed to a maximum over 120 to 240 seconds[154]. These effects were mediated by both β_1-AR and β_2-AR[153;154] and the increase in cAMP persisted for up to 15 minutes[146;228], similar in time scale to that of the TCC data presented herein.

Ligand binding of β-AR-$G_{\alpha s}$ stimulates PKA/ cAMP causing phosphorylation of serine and threonine residues on the α_{1c} and β_{2a} subunits of L type calcium channels augmenting I_{CaL} via an increased mean channel opening time and an increased P_o[90;238;239;302-307]. Calcium entry into the cytosol acts as a negative feedback to I_{CaL} although other gating modalities such as voltage inactivation also occur[161]. Although there are at least three types of β-AR expressed in the human heart, little is known of the physiological role of the β_3-AR in the atrium and discussion in this work will be limited to β_1-AR and β_2-ARs[308]. In human atrial myocytes β_1-ARs couple to solely to $G_{\alpha s}$ stimulating I_{CaL} via PKA and cAMP, however β_2-ARs couple to both $G_{\alpha s}$ and $G_{\alpha i}$ which can exert negative feedback on I_{CaL} and other cellular processes activated by β_2-AR stimulation[232;309-313]. PKA is anchored to the plasmalemma, co-localising with the L type calcium channel and being bound by PKA anchoring protein (AKAP) or AHNAK protein[314;315].

No data comparing I_{CaL} kinetics obtained using the differing methodologies has been published to date. The purpose of the comparison in this project was to establish if the formation of a ruptured patch with nystatin present in the pipette would preclude further analysis of I_{CaL} so recorded because of the possible confounding effect of nystatin entering the myocyte cytosol via a ruptured patch.

The increased duration of stable I_{CaL} recordings with nystatin perforated patch recordings is consistent with the published literature[220;221] and confirmed the possibility of subsequently performing further yet more complex experiments.

It has been shown that cardiac β-AR function decreases with increasing age in humans[316;317]. Mean β-AR density, the ratio of β_1-AR: β_2-AR and $G_{\alpha s}$ activity was unchanged[157], however, $G_{\alpha i}$ levels increase in human right atrium in association with ageing and correlate with a significant reduction in adenylyl cyclase activity in response to mixed β_1-AR: β_2-AR agonists resulting in a ten fold increase in the EC_{50} of the positive inotropic response to ISO[157]. Animal models of ageing have demonstrated that reduced responsiveness to β-AR stimulation in isolated myocytes is due to reduced receptor: ligand: second messenger activity with preserved calcium handling protein apparatus[231]. The lack of effect of increasing age on I_{CaL} could be explained by a significant reserve in the number of L-type calcium channels available for opening or an age related decrease in the activity of PDE4 which transduces the $G_{\alpha i}$ signal to inhibit cAMP. This possibility highlights the potential role of second messenger sub-systems and compartmentalisation within the atrial myocyte to separate I_{CaL} acting as a trigger for excitation contraction coupling from an adrenergic inotropic effect of ISO[38;318-321]. Alternatively the lack of an age related effect may represent a type 2 error due to the homogeneity of our sample of patients or indeed provide evidence of significant reserve of second messenger coupling to I_{CaL} such that increased inward current in response to ISO is maintained despite the reduction in $G_{\alpha s}$: $G_{\alpha i}$ ratio present in myocytes isolated from senescent patients.

Gender has been associated with altered repolarisation but no reports of altered human I_{CaL} have been published to date[298]. Data from this laboratory and others have consistently failed to identify any significant difference in I_{CaL} characteristics

in human atrial myocytes from male as opposed to female patients[79;144;223;281;284;287;322].

Pre-operative drug therapy had no discernible effect on I_{CaL} under control conditions nor on the adrenergic effect of ISO. Although chronic β-B therapy has been associated with reductions in APD_{90} and ERP these were related to reductions in I_{to} in the absence of an effect on I_{CaL}[144]. These electrophysiological changes, termed “pharmacological remodelling”, were associated with a reduced occurrence of AF_{pcs}, but were identified in vitro, independent of receptor occupancy[144].

Although a third, and possibly a fourth β-AR have recently been identified, little is known of their physiological role in the heart[308;323-325]. All subtypes of human myocardial β-AR are guanine nucleotide protein receptors which bind Norepinephrine undergoing conformational change which in turn activates membrane bound heterotrimeric G proteins[326-328]. The ratio of β_1-AR to β_2-AR expression in human right atrium is controversial with several reports suggesting that the ratio varies between 65:35 to 80:20[145;229;301;329;330]. β_1-AR activation with Norepinephrine and β_2-AR binding with Epinephrine can equally maximally increase inotropy in human atrial myocardium[152;331;332], and the β_2-AR selective agonist zinterol demonstrates that both β-AR receptors are coupled to the $G\alpha_s$ / adenylyl cyclase/ cAMP/ PKA/ PLB phosphorylation/ TnI phosphorylation/ TnC phosphorylation system resulting in positive inotropic and lusitropic effects[333].

Chronic β_1-AR selective β-B therapy sensitised β_2-AR in human left and right atrial myocytes[334-339] resulting in an enhanced inotropic action of mixed and selective β_2-AR agonists in the absence of β_1-AR antagonists suggestive of a persistent modification of receptors and intracellular messenger systems[152;337].

However, human atrial β_2-AR hyper-responsiveness of up to a 10 fold increase[152] cannot be explained by increased receptor density alone[159;301;340]. Following β-B therapy in tissue samples[335] and myocytes marked increases in β_2-AR responsiveness[152;331;337] and arrhythmia[290] were observed. The atrial inotropic response to NE via β_1-AR [152;331;337] and E[341] was not enhanced following chronic BB therapy, however chronic β-B therapy was associated with noradrenalin evoked 'arrhythmia' mediated via β_1-AR[290].

Chronic pretreatment with β-B therapy may[330;334;335;337] or may not[324] increase β-AR expression in human atrial myocytes. Selective β_1-AR blockade has been shown to enhance the activity of $G_{\alpha s}$ protein in human atrial tissue[160;334] and the sensitivity of β_2-AR[152;338] without any demonstrable increase in numbers of β-AR or of cAMP responsiveness[152;342;343], is suggestive of enhanced coupling of the β-AR to adenylyl cyclase via $G_{\alpha s}$ or perhaps an inhibition of PKA phosphorylation of $G_{\alpha s}$. Wang et al[160] among others measured the α subunit of $G_{\alpha s}$ and $G_{\alpha i}$ and the β subunit of G proteins expression at protein and mRNA levels and found no significant difference in human atria from BBY v BBN patients, however $G_{\alpha s}$ function was found to be significantly (2 fold) enhanced in BBY patients.

There are 4 types of adenylate cyclase found in human atrium (IV-VII)[343]. β-AR activation rapidly activates BAR kinase which phosphorylates β-AR resulting in subsequent loss of G-protein coupling[343] and there was no significant difference in the absolute levels of gene expression among adenylate cyclase subtypes in BBY v BBN. As the inotropic effects of synthetic cAMP[152] or forskolin[291] are not augmented following chronic β-B therapy then perhaps a possible role for altered amplification of receptor coupling via G_s proteins exists[344].

Desensitisation of β-AR occurs via a family of serine threonine kinases called GPRC Kinases (GPRCK) abbreviated to GRK- GRK the ones specific to β-AR are named βARK. These are similar in structure to other serine threonine kinases such as PKA and PKC. When the receptor is phosphorylated by the βARK the β-AR is desensitised[345]. GRK2 is βARK1 and GRK3 is βARK2 and in the myocardium they only recognise and thus phosphorylate agonist occupied receptors[346]. The $G_{\beta\gamma}$ subunit which remains from the 'splicing' of the G protein complex then interacts with the carboxyl terminal of the cytosolic βARK resulting in the translocation of the GRK to the membrane where it phosphorylates the activated receptor[347]. Desensitisation in the presence of persistent agonism such as catecholamine elevation in heart failure (termed homologous desensitisation) requires not only GRK but also β-arrestin which prevents $G_{\alpha s}$ coupling to the catecholamine bound β-AR[348]. Reduced β-AR density and function in failing hearts is in contrast to βARK which is significantly increased in patients with CHF[349], hypertension[350] and myocardial ischaemia[351]. The premise that elevated βARK1 is protective is being suggested by in-vitro studies, as reduced βARK expression levels post myocardial infarction have been associated with an increased inotropic response to ISO[352] - analogous to the interruption of neurohumoural remodelling of CHF by β-B therapy- indeed β-B therapy is associated with a reduction in βARK1, β-AR downregulation increasing agonist sensitivity and improving cardiac function in vivo[353]. As β-ARK preferentially phosphorylates the β_2-AR rather than β_1-AR[354], yet another possible mechanism for selective inactivation of the β_2-AR exists. β_1-AR blockade with selective β-B therapy will inhibit β-ARK activity in β_2-AR resulting in more non-phosphorylated β_2-AR as a result of background autonomic tone.

The lack of effect of chronic CCB therapy on beta adrenoceptor density is less controversial. Published reports confirm that CCB has no effect on adrenoceptor density in human right atrium[330;335;337;355;356]. Two previous reports in humans

described a decrease in I_{CaL} associated with chronic pre-operative CCB therapy[155;282], however a larger study comprised of data from this laboratory and three other investigators found no such association[144;223;281;287]. In animal models most studies reported no association between chronic CCB therapy and I_{CaL} and indeed in one study an increase in I_{CaL} was described[357-360]. It is not clear why the difference in results from human atrial myocytes occurred, however the initial reports described results with significant methodological differences between the studies.

There has been only one published report of the acute effect of the ACE-I drug, enalapril, to increase I_{CaL}[282], but the present study is in total agreement with four previously published reports demonstrating no association with chronic ACE-I therapy and altered I_{CaL} characteristics[144;223;281;287].

There have been no studies published to date demonstrating an association between pre-existent hypertension and altered human atrial I_{CaL} characteristics. If true, one possibility, discussed above, is elevated levels of βARK[350]. At first glance this would seem counterintuitive as reduced levels of βARK were associated with an increased inotropic response to ISO[352]. However there may indeed be a confounder at play. Most patients from whom we received atrial tissue were undergoing cardiac surgery for CHD and were receiving chronic β-B therapy which is associated with a reduction in βARK1, increasing agonist sensitivity in vivo[353]. In retrospect, pooled data from our own laboratory, with a much larger sample size and following multivariate analysis similarly does not identify any such association between pre-existent hypertension and altered human atrial I_{CaL} characteristics[144;284], suggesting that this is a type 1 error due to the small sample size.

Although the L-type calcium current recorded in canine ventricular myocytes from the infarct border zone is reduced with altered kinetics and response to adrenergic stimulation following MI in vitro[361-363], no such association has been documented in human atrial myocytes[144;284].

The acute effects of sympathomimetics are blunted in patients with CHF and this has been thought to be related to any or all of the following effects: a CHF induced down regulation of β-AR, internalisation of the receptor ligand complex with out downstream cellular effects, depletion of intracellular stores of secondary messengers or impaired phosphorylation of intracellular messengers[126;364;365].

The positive inotropic and lusitropic effects of β_1-AR and β_2-AR ligand binding persists in human atrial tissue from failing hearts[152;331-333]. Despite similar levels of expression of β_1-AR and β_2-AR[339], however, the effects of β_2-AR binding E are preserved but the β_1-AR mediated inotropic effect of NE was reduced fourfould in tissue preparations from patients with CHF with no change in lusitropy. Reports of reduced I_{CaL} response to β-AR stimulation are controversial, and may reflect altered β-AR availability and response in myocytes from CHF patients rather than impaired intracellular signal transduction mechanisms[114;279;366;367]. This hypothesis, that β_2-AR linked to $G_{\alpha i}$ proteins[232;309] may oppose the effects of $G_{\alpha s}$ stimulation in human atria, particularly in patients with CHF[312], due to the relative downregulation of β_1-AR as compared to β_2-AR[342] remains unsubstantiated[330;332;340;342]. One report has suggested that the ISO induced stimulation of adenylate cyclase activity was weaker in atrial tissue compared to ventricular specimens from failing hearts[301]. There appeared only a weakly positive correlation of increasing β-AR receptor concentration and adenylate cyclase activity in right atrial specimens[301]. Very recently human atrial myocytes with very small detectable “down-regulated” I_{CaL} at baseline from patients in sinus rhythm suffering from LVSD, said to be at high risk

of developing AF, were observed to display an exaggerated response to stimulation with ISO[299]. This is on stark contrast to the current work and pooled data from our laboratory performed under more physiological conditions.

We, and others, have found, following multivariate analysis, that there were no detectable differences in I_{CaL} peak current, I: V relationship or kinetics under basal conditions nor following stimulation with ISO at the approximate EC_{50} (50 nM) between cells from patients with as opposed to without LVSD[144;279;284;300;368].

Some of the original studies examining inward calcium current in human atrial myocytes linked calcium overload via increased I_{CaL}, either alone or as a function of tachycardia, to the subsequent development of AF[81;82;104;369]. It is widely accepted that the response to AF, that is the electrophysiological remodelling that occurs as a result of the presence of AF or as a result of the pathophysiology which has also allowed AF to supervene is associated with an approximate 70% reduction in I_{CaL}[69;79;82;98;100;370]. In contrast to early data which demonstrated an association between large I_{CaL} and post-operative AF, but not the magnitude of an adrenergic increase in I_{CaL}[81], Dinanian et al[299] report that the smaller the magnitude of baseline I_{CaL} the greater the adrenergic response to ISO. However no information regarding the subsequent development of AF_{pcs} was available. In contrast neither the baseline magnitude of I_{CaL} nor the adrenergic response to ISO was predictive of subsequent AF_{pcs} in this study nor in pooled data from this laboratory[284].

3.6.4 Anti-adrenergic effect of acute superfusion with ET-1

ET-1 alone, a mixed $ET\text{-}_A$ and $ET\text{-}_B$ receptor agonist, at the maximal concentration tested (10 nM), had no discernible effect on peak I_{CaL}. Similarly, 10 nM Sarafatoxin

S6-c, a selective ET-$_B$ receptor agonist had no significant effect on peak I_{CaL} indicating that there was no apparent effect rather than a balance of two opposing effects on peak I_{CaL}. In contrast, ET-1, in a concentration dependent fashion from 0.01 to 10 nM, had an anti-adrenergic effect on peak I_{CaL} prestimulated by 0.05 μM ISO. This anti-adrenergic effect was partially reversible, both by washout and also by either *a priori* or co-application with the selective ET-$_A$ receptor antagonist FRA at 10 nM. Sarafatoxin S6-c at 10 nM had no effect on prestimulated peak I_{CaL}, nor did it prevent the anti-adrenergic effect of 10 nM ET-1 on peak I_{CaL} which had been pre-stimulated with 0.05 μM ISO.

In human atrial myocytes, ET-1 alone was found to increase[199], decrease[199;200] or have no effect[199;200] on I_{CaL} under control conditions. It is not immediately apparent why this should be. Initial reports in animal models described stimulatory[196;248;258;259], inhibitory[195;196;248;260-263] or no effect[203;247;249;264] of ET-1 directly on I_{CaL}, with an EC_{50} between 1 and 10 nM, mediated via ET-$_A$ receptors[195;196;203;248;249;258-264]. In addition to the possible direct effects on I_{CaL}, activation of NHE[265] and NCX[266-268] via PKC have also been described, but such activation would be expected to reduce I_{CaL} as a result of calcium induced inactivation which was not observed.

ET-1 has been noted to inhibit I_{K1} via PKC in human atrial myocytes at physiological temperature using whole cell patch clamp[371] resulting in a hyperpolarised resting membrane potential[270;272]. ET-1, at a concentration observed in patients with severe CHF[372], induced maximal inhibition of outward K^+ and NCX currents in human atrial but not ventricular myocytes similar to experience in animal models[268;271;273;373]. Whether these effects, not directly measured herein, could in some way compensate for a direct effect of ET-1 on I_{CaL} rendering the net effect to be zero cannot completely be discounted. However I_{CaL}

was recorded with caesium in the pipette blocking K^+ currents in the cells studied and the TCC experiments discount a time dependency of opposing effects reported[199;200].

Differing methodologies may explain apparently contradictory results. The requirement for ET-1 to convey it's signalling via temperature sensitive secondary messengers may result in a population of cells unable to respond conventionally depending on the level of oxidation or phosphorylation of the cell cytosol proteins[84;161;374-379]. A similar paradox of apparently opposing effects of ANP on I_{CaL} was resolved by ensuring the availability of second messengers, in this case cGTP, in the pipette during ruptured patch experiments[199;380;381].

Initial experiments investigating potential arrhythmogenic effects of ET-1 in animal models were contradictory confounded by the peptide's vasoconstrictor induced ischaemic pro-arrhythmic effects[54-60], however several groups began to identify acute electrophysiological effects apparently directly attributable to ET-1 in the absence of ischaemia[56;61-64].

Historically a clearer consensus began to emerge regarding ET-1 having a positive inotropic effect and influencing arrhythmogenesis in human tissues although no obvious mechanism was initially apparent[191]. Initially this apparent positive inotropic effect was assumed to be related to a direct augmentation of I_{CaL} by ET-1. However the positive inotropic effect of ET-1 has been demonstrated to occur in the absence of an increase in I_{CaL}[179;197;382-384] or the calcium transient suggestive of increased myofibrillar sensitivity to calcium[195;385;386]. In addition the effect of ET-1 via $ET\text{-}_A$ receptors in tissue preparations removed from failing hearts varied considerably and was thought to relate to altered ET-1 receptor expression rather than any changes in I_{CaL}[175;184;372;387;388].

The positive inotropic effect of ET-1 in human atrial preparations[175;177-179;188;193;387;388] has now been identified to be mediated by ET-$_A$ receptors via $InsP_3$/ DAG/ PKC[177;184;187;188;193;387;388] in the presence[179;188] and absence of any effect on NHE[177;388]. Of note is that the positive inotropic effect of ET-1 was apparently independent of patient age, gender, pre-operative drug therapy and LVSD which have previously been associated with altered GPCR status and I_{CaL}[386].

In animal models evidence exists for an additional three possible mechanisms by which ET-1 can exert a positive inotropic effect in cardiac myocytes in the absence of an augmentation of I_{CaL}: stimulation of NHE resulting in increased myocyte $[pH]_i$ sensitising troponin C to calcium, increasing the $[Na]_i$ resulting in mode reversal of NCX and PLC activation induced $InsP_3$ formation causing increased SR calcium uptake, storage and release[187;194;197;241;265;267;389-392].

The above experiments described the effects of acute exposure to ET-1, however one study has described the effects of chronic (≥5 days) exposure of rat myocytes to ET-1 which resulted in blunting of the positive inotropic effect of both ISO and increased $[Ca^{2+}]_{ec}$ mediated by ET-A, via PKC and NHE[393].

In one of the three studies examining the receptor mediated effects of ET-1 alone on I_{CaL}[199], an intrinsic stimulatory effect of ET-1 on I_{CaL} was prevented by pre-treatment of the atrial myocytes with the ET-$_B$ receptor agonist BQ-788. In human tissue BQ-788 has been shown to have low affinity for and little selectivity for ET-$_B$ receptors[394]. This observation could be explained by a lack of effect of ET-1 alone which would be consistent with that observed in the other two published reports[200;322]. No additional data other than I_{CaL} current density at baseline was provided to explain this apparent dichotomous effect[199]. This group have published

data obtained at room temperature with variable effects dependent on small initial I_{CaL} density without clearly identifying an obvious mechanism[92;299].

Evidence exists for a basal constitutive cAMP/ I_{CaL} activity level in human atrial myocytes[281;287;288;395] which is counterbalanced by type 1 and type 2A phosphatases (PP1 and PP2A)[91] and it may be that two populations of cells with differing phosphatase activity or perhaps altered basal levels of cAMP may explain the dichotomous results. In human right atrium ET-1 was demonstrated to have little or no effect on cAMP under basal conditions, but inhibited the action of adenylate cyclase to produce cAMP due to ET-$_A$ receptor/ $G_{\alpha i}$/ IP_3 formation independent of I_{CaL} and PKC[187;200]. Indeed the anti-adrenergic effect of ET-1 was abolished during simultaneous intracellular application of 8-Br-cAMP[200].

In human atrial myocytes, ET-1 alone was found to increase[199], decrease[199;200] or have no effect[199;200] on I_{CaL} under control conditions. Following pre-stimulation with ISO, ET-1 consistently had an anti-adrenergic effect mediated via ET-$_A$ receptors coupled to $G\alpha_i$[199;200]. Numerous groups have developed a theme of ET-1 whether via crosstalk in intracellular messengers or via direct antagonism demonstrates an anti-adrenergic effect on chronotropy, inotropy and I_{CaL}[245-249].

Adrenergically prestimulated I_{CaL} was inhibited by 10 nM ET-1 via ET-$_A$ receptors acting on $G_{\alpha i}$ (pertussis sensitive) GPCRs[245;247;262] which reduced cAMP via PDE in guinea-pig ventricular myocytes[262] and human atrial myocytes[200] in the absence of any direct effect on the L-type calcium channel itself[198;200]. Similar to human atrial I_{CaL}, ET-1 binding to ET-$_A$ receptors had no effect on basal I_{Cl} in animal models, however ET-1 with an EC_{50}~ 1 nM, activating $G_{\alpha i}$ had an anti-adrenergic effect reducing ISO prestimulated I_{Cl} via reducing intracellular cAMP (PDE)[205;273].

ET-1 binding to ET-$_A$ receptors on animal cardiac myocytes has a number of biological effects mediated via stimulation of $G_{\alpha i}$[396-398]. ET-1 had an anti-adrenergic negatively chronotropic effect mediated via ET inhibition of formation of cAMP both under basal conditions and following adrenergic stimulation with ISO[399] and ET-1 prevented apoptosis induced by 10 μM ISO downstream to the β-AR and $G_{\alpha i}$ induced alterations in cAMP mediated intracellularly via phosphatidylinositol and protein phosphate kinases[400]. As apoptosis is a recognized sequel following the development of AF or atrial dilation[96], whether ET-1 has a direct effect to promote apoptosis or has an anti-adrenergic effect to oppose apoptosis mediated by catecholamines remains to be determined.

The selective ET-A receptor antagonist FR139317 inhibited the binding of ET-1 to ET-A receptors in a concentration dependent, monophasic fashion with an IC_{50} of 0.53 nM in the absence of agonist activity[401]. The possibilities for ET-1 mediated anti-adrenergic effect on human atrial I_{CaL} are as follows: ET-1 stimulates calcium release from the SR reducing the electrochemical gradient producing calcium mediated inactivation of I_{CaL}[195;197;258], ET-1 stimulates IP_3 formation and in so doing inhibits cAMP formation by adenylate cyclase reducing the ability of cAMP dependent kinases to activate I_{CaL}[187;188;200;262;273;399], or that ET-1 stimulates cyclic nucleotide phosphodiesterase via cGMP hydrolyzing cAMP antagonizing the adrenergic effect of β-AR stimulation[187;199;200;242] on I_{CaL} but not NCX[402;403]. The apparent contradiction in an inhibitory effect on I_{CaL} but a positive inotropic effect could be explained by the relative efficacy of the differing second messenger systems effected by ET-1 binding to ET-$_A$ receptors: sensitising the SR and increasing cytosolic calcium during diastole will oppose calcium entry via I_{CaL}[195;197;258], PLC activation and IP_3/ DAG stimulation produces a positive inotropic effect but the associated inhibition of cAMP will reduce I_{CaL}

amplitude[187;188;200;262;273;399] and activation of NHE[265] and NCX[266-268] via PKC will result in a positive inotropic effect with little or no direct inhibitory effect on I_{CaL}.

If one accepts that ET-1 is a positive inotrope but a negative lusitrope, it is accepted that the PIE of ET-1 differs from that of beta-adrenergic agonists such as isoproterenol which is positively inotropic but also positively lusitropic. ET-1 increases the calcium transient and is thought to do this via NHE and I_{CaL}[404]. Stimulation of NHE causes intracellular alkalinisation and an increase in intracellular Na[265;405;406] which reverses the normal direction of the sarcolemmal NCX and thus increases intracellular calcium[407]. This may explain why for a given PIE ET-1 requires a smaller increase in intracellular Ca and also the different lusitropic effects when compared to beta adrenergic agonists[407]. Additionally alkalinisation of the cardiac myocytes increases the affinity of troponin C for calcium, sensitising actomyosin ATP-ase to calcium resulting in a positive inotropic effect and decreases the rate of dissociation of calcium from the troponin C-ca complex resulting in negative lusitropy.

4 RESULTS (ii)

Single atrial myocyte action potential studies

4.1 Introduction and aims

The primary function of the heart is to pump blood around the body. In order for this to occur effectively a coordinated cycle of excitation, contraction and relaxation in cardiac myocytes must occur. The action potential is a time dependent integration of multiple ionic currents present on the cell membrane which not only initiates intracellular excitation-contraction coupling but also provides the excitatory stimulus for adjacent myocytes. This electrical stimulus, when normally propagated around the heart, permits coordinated 3-D contraction forcing blood through the cardiac chambers and out into the blood vessels.

In human atrial myocytes action potentials are generated by the rapid inward influx of fast sodium current (I_{Na}) depolarising the cell membrane. This depolarisation opens long lasting L-type calcium channels (I_{CaL}) permitting calcium to enter the cell triggering mechanical activation of the contractile apparatus within the cell and forming the electrical plateau phase characteristic of cardiac myocytes to oppose rapid repolarisation by potassium currents (I_{TO}). Action potential repolarisation electrically resets the myocytes in a rate dependent manner permitting the electrical cycle to be repeated and the ensuing refractoriness ensures sufficient time for intracellular processes to complete in time for the subsequent action potential.

Propagation of an action potential between neighbouring myocytes transmits stimuli from one region of the heart to another. The action potential is modulated by the physiological environment surrounding the cell, the cell membrane voltage, the membrane ion channels and intracellular regulatory molecules in a complex fashion. Up until this point we have limited our investigation to one ion channel, I_{CaL}. It is important not only to place the effects of ET-1 on I_{CaL} characteristics in context within the action potential but also to translate these changes into myocyte functional properties, that is repolarisation and refractoriness, in addition to identifying any other changes in action potentials which may indicate the possibility of ET-1 effecting cardiac ion channels other than I_{CaL}.

The effect of catecholamines to elevate the plateau phase of mammalian cardiac action potentials were described in detail many years ago[227;408-412]. Although the cellular basis of this plateau elevation took some time to fully be investigated it became clear that this adrenergic effect was the result of cAMP mediated increased conductance of I_{CaL}[227;408-416]. However, despite these numerous reports, relatively little has been published in detail regarding the effect of ISO specifically on human atrial isolated myocyte action potentials at physiological temperature[161].

Initial experiments investigating potential arrhythmogenic effects of ET-1 in animal models were contradictory, confounded by the peptide's vasoconstrictor induced ischaemic pro-arrhythmic effects[54-60]. However, several groups began to identify acute electrophysiological effects apparently directly attributable to ET-1 in the absence of ischaemia producing focal activity[56;61-64].

Endothelin has been reported to have a direct effect on I_{Na}[176;200;261], I_K[62;269-272;417], I_{CaL}[195;196;248;258-261;263;399], I_{Cl}[273], NHE[265] and NCX[266-268] ionic currents in cardiac myocytes in various animal and human studies. In contrast, very little is known

about the effects of ET-1 on human atrial myocyte action potentials. In this report we have described an absence of effect of ET-1 alone on I_{CaL} but observed powerful anti-adrenergic effects of ET-1 to completely reverse the effect of ISO on I_{CaL} in human atrial isolated myocytes.

Adrenergic stimulation is recognised to be pro-arrhythmic in both human atrium and ventricle via automaticity, triggered activity and reentrant mechanisms[69;162;418]. What is not known is whether ET-1 would modify human atrial myocyte action potential characteristics in such a way as to be anti-arrhythmic, as suggested by its effect on I_{CaL}, or if it would be observed to have other pleiotropic effects which could promote arrhythmogenesis.

In view of the fact that initial reports described stimulatory[196;248;258;259], inhibitory[195;196;248;260-263] or no effect[203;247;249;264] of ET-1 on I_{CaL} we wished to confirm whether the anti-adrenergic effects of ET-1 on I_{CaL} would translate into alterations in action potentials. In addition, using concentrations similar to those observed to have electrophysiological effects in animal models[195;196;203;248;249;258-264] and reported to be present in human patients suffering from CHF[123;125;128;255] we hoped to gain some mechanistic insight into arrhythmogenesis in heart failure.

The aims of these experiments were as follows:

- To establish that human atrial isolated myocyte action potentials could be reliably recorded using the nystatin perforated patch clamp technique under “physiological” conditions
- To investigate whether patient characteristics, chronic pre-operative drug therapy or pre-operative comorbidity was associated with altered electrophysiological properties such as depolarisation, repolarisation and refractoriness.

- To determine the effects of ISO, at the EC_{50} previously demonstrated to reliably augment I_{CaL}, on action potential characteristics and spontaneous depolarisations.
- To investigate the effect(s), if any, of ET-1 on action potential characteristics and spontaneous depolarisations.
- To compare and contrast the effect(s) on action potential characteristics of ET-1 and ISO in combination.

4.2 Methods

Myocytes were isolated and superfused with solutions as previously described (See Methods 2.3.1). Experiments were performed in current-clamp mode whereby a constant or pre-specified time-varying current is applied to the myocyte and the resultant change in membrane potential, the action potential, is recorded (See Methods section 2.4.1).

Myocytes in the resting state were current clamped to -80± 5 mV and subsequently the holding current was maintained <150 pA thereafter. Action potentials were stimulated at 75 beats per minute (bpm) using 5 ms current pulses of 120% threshold strength.

The APD was calculated as the interval between the action potential upstroke and repolarisation to the level of 50% (APD_{50}), 75% (APD_{75}) and 90% (APD_{90}) of the upstroke amplitude in all cells (See Figure 4.1). Additionally, in order to further investigate the effect of CCB drugs action potential repolarisation at 60% (APD_{60}) was also calculated. A representative example of the measurements made to the raw current tracings to calculate phase 0 characteristics and repolarisation (Figure 4.1A) and refractoriness in Figure 4.1B.

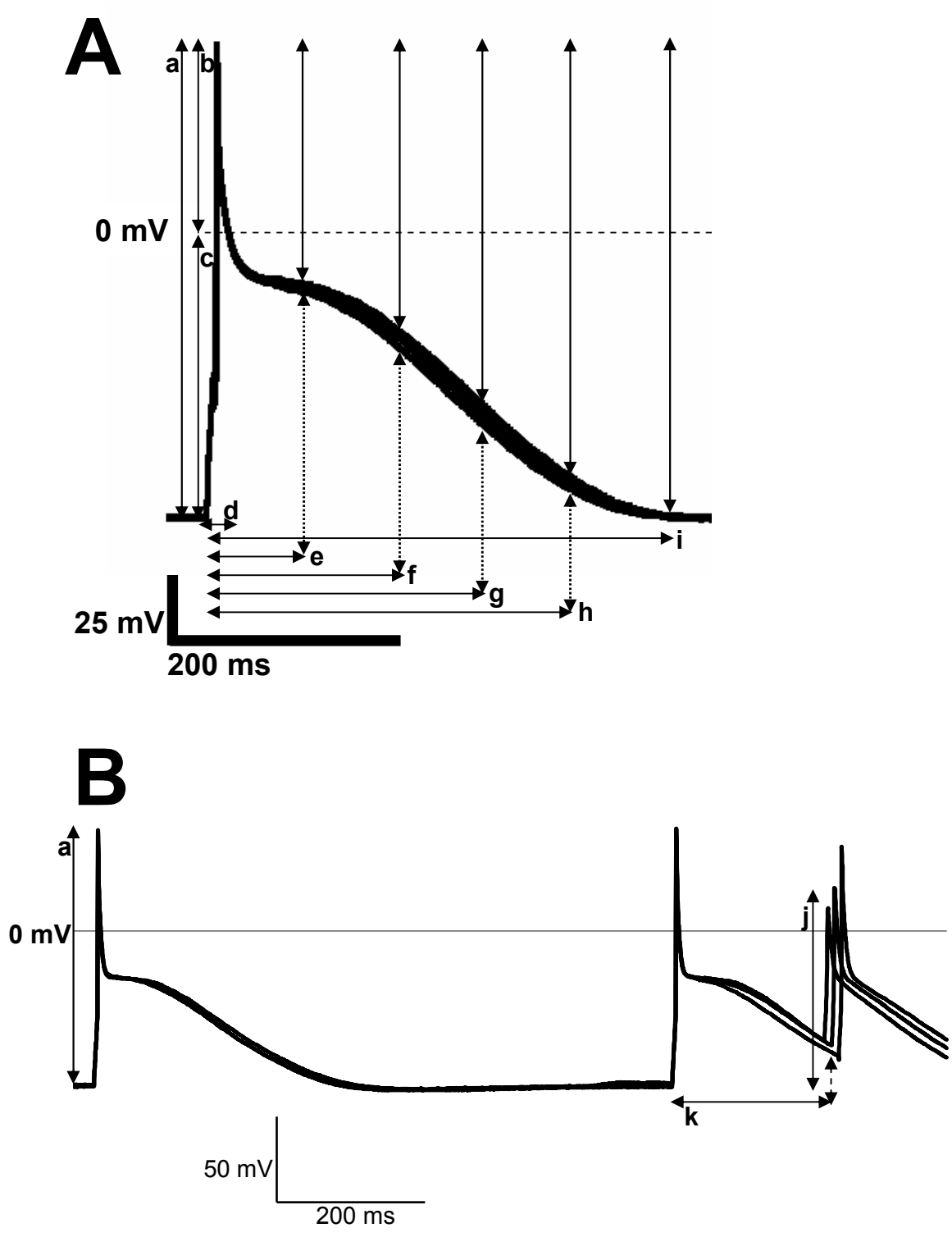


Figure 4.1 Diagrammatic representation of measurements of A) repolarisation and B) refractoriness in human atrial isolated myocyte action potential recordings. a= amplitude, b= overshoot, c= MDP, d= time to peak amplitude (a/d= V_{max}), e= APD_{50}, f= APD_{60}, g= APD_{75}, h= APD_{90}, i= full repolarisation, j= 80% amplitude threshold, k= ERP.

4.3 Protocols

Both ERP and REP protocols were performed under control conditions once an appreciation of the occurrence of spontaneous depolarisations was realised (See Methods 2.4.1). Both ERP and REP protocols were performed under control conditions and subsequently acute superfusion with ISO at 0.05 μM or 10 nM ET-1 alone was performed as soon as stable action potential recordings (ERP and REP) had been completed. Following exposure to ISO or ET-1 for 90 seconds and 3 minutes respectively, based on time to peak effect on earlier I_{CaL} experiments and assuming stable action potential recordings were observed, the addition of other subsequent reagents to the superfusate occurred. A diagramatic representation of action potential experiment is given in Figure 4.2.

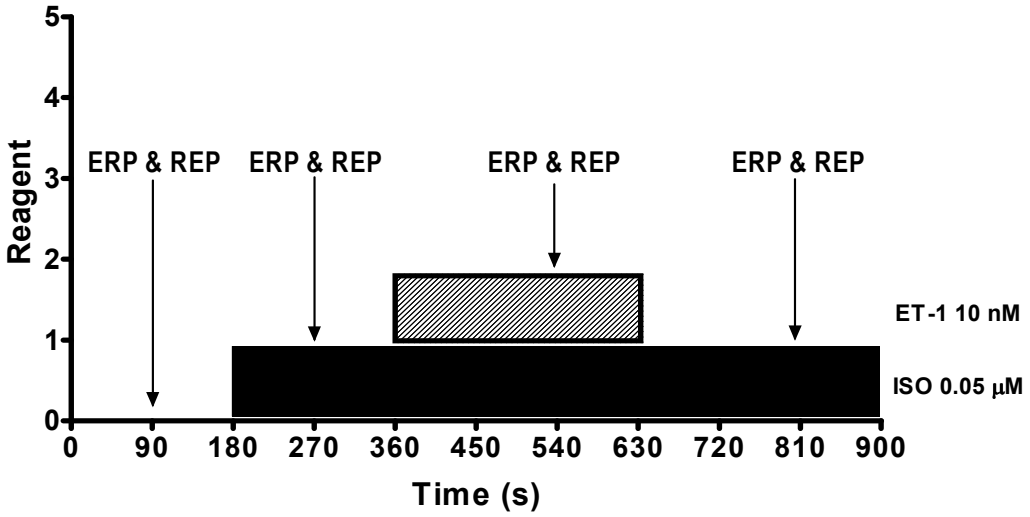


Figure 4.2 A representative schema of the order and timing of recordings of human atrial isolated myocyte action potentials made during acute superfusion with both 0.05 μM Isoproterenol and 10 nM ET-1 in combination.

4.4 Statistics & data analysis

Data were recorded and analysed off-line as described in Methods section 2.5.1.. The action potential characteristics of the entire dataset followed a normal distribution and therefore the paired Student's t test was employed to determine

the degree of difference between sub-analysed samples under control conditions. This test was selected as it is the most valid, accepted test with which to assess the probability of a similar or more extreme result being arrived at by chance in samples of small size[79;144;161;223]. Where a comparison between two differing populations of data are presented the variance of the two groups has been assessed to permit the use of the unpaired Student's t test was employed. As previously stated this report is based on all available information in order to maximise utility.

4.5 Results

4.5.1 Human atrial myocyte action potential morphology and phase 0 characteristics under control conditions

Action potentials were characterised by rapid upstrokes with depolarisation consistently reaching positive potentials (overshoot). We recorded only type 1 and 2 action potentials demonstrating a brief rapid repolarisation phase followed by a plateau phase prior to full repolarisation back to the diastolic potential. In order to quantify differences in action potential morphology a number of measurements of phase 0 characteristics, repolarisation and later, refractoriness, were made. The numerical values of the various electrophysiological characteristics are represented in Table 4.1. A representative action potential recording is displayed in Figure 4.3.

Cellular action potential characteristics followed a normal distribution. In order to determine whether samples from a particular patient were consistently more than 2 SD outwith the single myocyte distribution curve patient means were also calculated and comparison with mean data were made. No cells from any patient required to be excluded. There was no significant difference in the maximal

diastolic potential (MDP) nor of phase 0 of the action potential depolarization characteristics of single myocytes as compared to patient mean (Figure 4.4 A-D). The mean MDP of all myocytes following hyperpolarisation was -83.62± 0.6 mV as compared with -83.35± 0.7 mV (n= 78 cells from 20 patients, mean± s.e.m., Figure 4.4A). The mean maximal rate of depolarization (V_{max}) observed in cells was 208.7± 5.6 V/s which was not significantly different from patient mean 206.8± 6.8 V/s (n= 78 cells from 20 patients, mean± s.e.m., Figure 4.4B). The action potential overshoot was similar regardless of cellular or patient mean: cell mean 60.7± 0.9 mV compared to 60.6± 1.2 mV (n= 78 cells from 20 patients, mean± s.e.m., Figure 4.4C). As expected the total amplitude of the phase 0 response of the action potential did not significantly differ between cells or patients. Mean amplitude of phase 0 response recorded from cells was 144.6± 0.9 mV and was 143.8± 1.2 mV (n= 78 cells from 20 patients, mean± s.e.m., Figure 4.4D) from patients.

4.5.2 Action potential duration under control conditions

Early, mid and late repolarisation are referred to as APD at 50% 75% and 90% respectively calculated as indicated in Figure 4.1 using phase 0 upstroke amplitude as the denominator. Action potentials were not triangular with an obvious plateau during early and mid repolarisation. In an attempt to confirm that cells were similar between and within patients a comparison between cell and patient means was performed and, as with MDP and phase 0 characteristics no significant difference was observed (Figure 4.5).

Mean action potential repolarisation at 50% (APD_{50}) observed from cells was 20.8± 2.6 ms compared to patient mean of 21.4± 3.5 ms (n= 78 cells from 20 patients, mean± s.e.m., Figure 4.5A). Action potential repolarisation at 75% (APD_{75}) observed from cells was 144.4± 7.8 ms and in patients was 147.7± 9.4 ms

(n= 78 cells from 20 patients, mean± s.e.m., Figure 4.5B). Similarly action potential repolarisation at 90% (APD_{90}) observed from cells was 216.3± 9.3 ms compared to mean patient APD_{90} of 215± 9.8 ms (n= 78 cells from 20 patients, mean± s.e.m., Figure 4.5C).

4.5.3 Effective refractory period (ERP) under control conditions

There were no significant differences observed between cellular atrial myocyte refractoriness and patient mean ERP recorded in 10 ms increments (Figure 4.6). The mean cellular ERP was 210.0± 8.1 ms compared to a patient mean of 213± 8.8 ms (n= 74 cells from 20 patients, mean± s.e.m., Figure 4.6A). The relationship of refractoriness to repolarisation (ERP/APD_{90}) was similar between cells and patients: mean myocyte ERP/APD_{90} ratio of 1.007± 0.03 compared to 1.042± 0.05 (n= 74 cells from 20 patients, mean± s.e.m., Figure 4.6B).

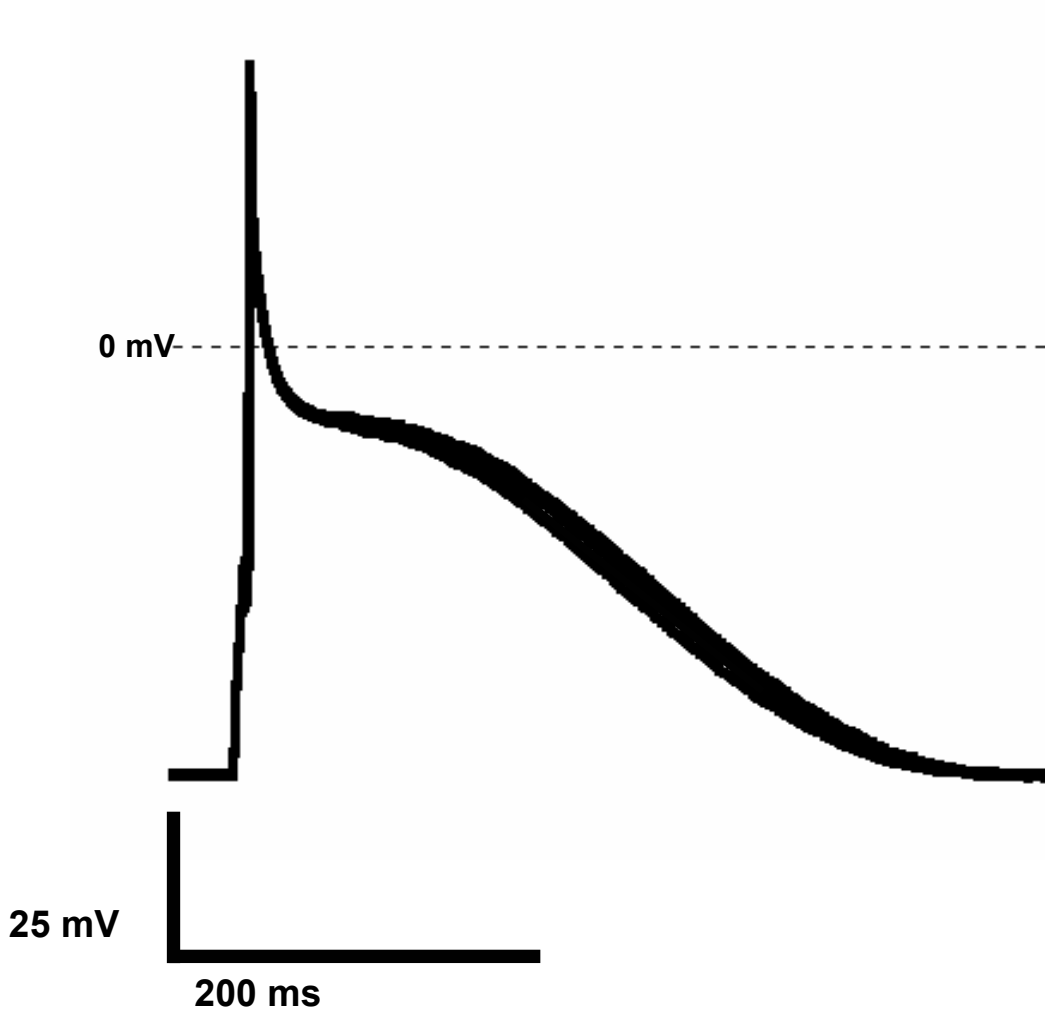


Figure 4.3 Representative human atrial isolated myocyte action potential recording made under control conditions.

	Parameter	Mean data	Published range to 2008
Phase 4	MDP	-83.62 ± 0.6 mV	-71 to -86 mV
Phase 0	V_{max}	208.7 ± 5.6 V/s	149 to 244 V/s
	Overshoot	60.7 ± 0.9 mV	48 to 53 mV
	amplitude	144.6 ± 0.9 mV	>90 mV
Repolarisation	APD_{50}	20.8 ± 2.6 ms	12 to 62 ms
	APD_{75}	144.4 ± 7.8 ms	118 to 138
	APD_{90}	216.3 ± 9.3 ms	176 to 447 ms
Refractoriness	ERP	210 ± 8.1 ms	184 to 233 ms
	ERP/ APD_{90}	1.007 ± 0.03	

Table 4.1 Comparison of mean action potential characteristics data under control conditions from this study (n_c=78, n_p=20 for phase 0 and repolarisation data, n_c=74, n_p=20 for refractoriness data) with reported ranges in the literature (see text for references).

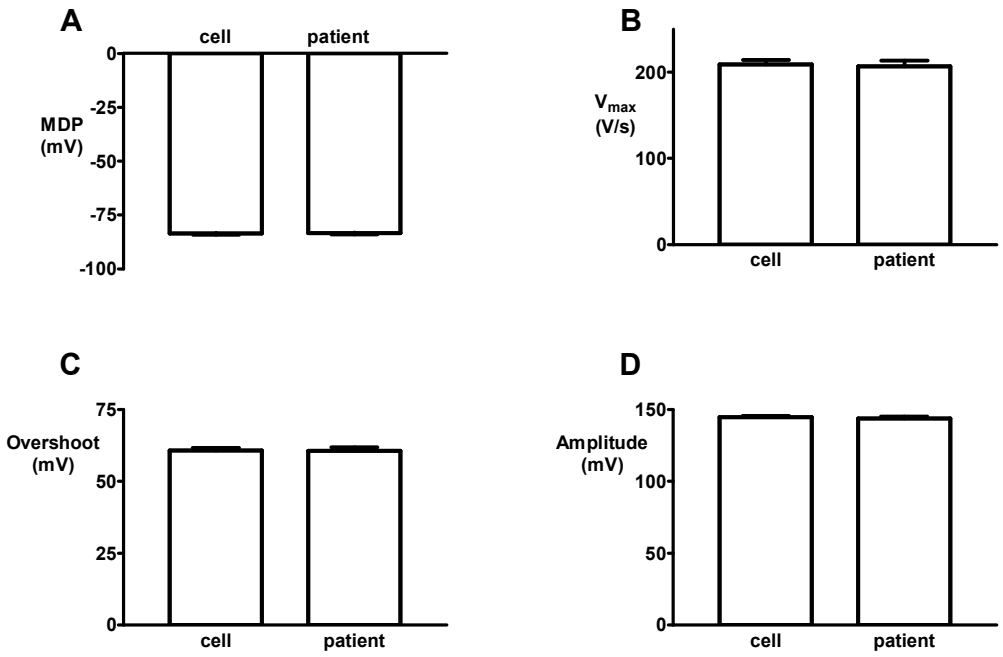


Figure 4.4 Comparison of cell mean with patient mean of phase 1 of the action potentials recorded under control conditions. A) Mean Diastolic Potential. B) upstroke maximum velocity. C) action potential overshoot. D) action potential amplitude (n= 78 cells from 20 patients).

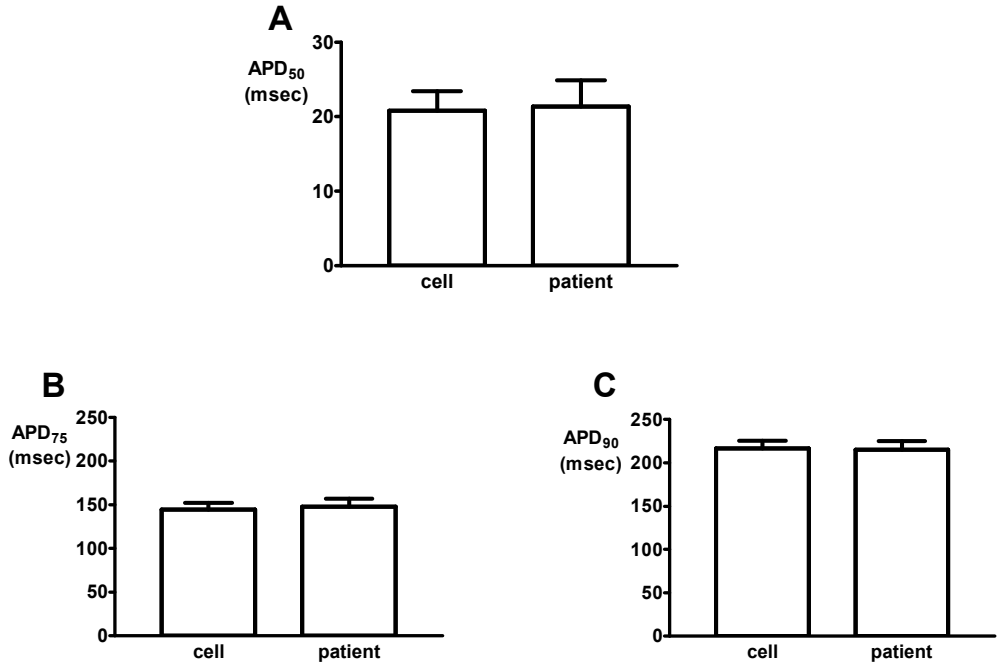


Figure 4.5 Comparison of cell mean with patient mean action potential duration. A) Action potential duration at 50% repolarisation (APD_{50}) B) Action potential duration at 75% repolarisation (APD_{75}) C) Action potential duration at 90% repolarisation (APD_{90}) (n= 78 cells from 20 patients).

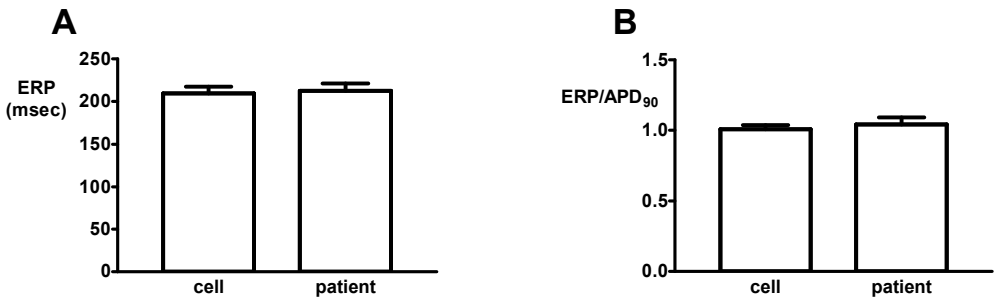


Figure 4.6 Comparison of cell mean with patient mean refractoriness. A) Effective refractory period (ERP) B) Ratio of ERP to APD_{90} (n= 74 cells from 20 patients).

4.5.4 Impact of methodology on atrial myocyte action potentials and refractoriness

4.5.4.1 Lack of effect of method of electrical access to the cell on action potential characteristics relating to depolarisation

There was no significant difference in the maximal diastolic potential (MDP) nor of phase 0 of the action potential depolarization characteristics of those myocytes in which the seal was obtained via a ruptured patch as compared with those cells in which the seal was a perforated patch. The mean MDP obtained with a ruptured seal was -85.1± 0.7 mV (n= 10 cells from 7 patients, mean± s.e.m.) as compared with -83.4± 0.7 mV (n= 68 cells from 13 patients, mean± s.e.m.). The maximal rate of depolarization (V_{max}) observed in cells in which a ruptured seal was obtained was 211.8± 14.9 V/s (n= 10 cells from 7 patients, mean± s.e.m.) which was not significantly different from those cells in which a perforated seal was obtained 208.3± 6.1 V/s (n= 68 cells from 13 patients, mean± s.e.m.). The action potential overshoot was similar regardless of the method of electrical access: ruptured seal formation was associated with a mean overshoot of 59.4± 3.0 mV (n= 10 cells from 7 patients, mean± s.e.m.) compared to 60.9± 0.9 mV (n= 68 cells from 13 patients, mean± s.e.m.). Consistent with these results the total amplitude of the

phase 0 response of the action potential did not significantly differ according to the method of electrical access to the cell. Mean amplitude of phase 0 response recorded from cells via a ruptured patch was 144.8± 2.7 mV (n= 10 cells from 7 patients, mean± s.e.m.) and was 144.6± 0.9 mV (n= 68 cells from 13 patients, mean± s.e.m.) from cells via a perforated patch.

4.5.4.2 Lack of effect of method of electrical access to the cell on repolarisation characteristics

There was no significant difference in the action potential repolarisation characteristics of those myocytes in which the seal was obtained via a ruptured patch as compared with those cells in which the seal formed was perforated. Action potential repolarisation at 50% (APD_{50}) observed from cells in which the seal ruptured during formation was 24.6± 8.5 ms (n= 10 cells from 7 patients, mean± s.e.m.) compared to 20.2± 2.8 ms (n= 68 cells from 13 patients, mean± s.e.m.) that observed from cells in which the seal formed via perforation. Action potential repolarisation at 75% (APD_{75}) observed from cells in which a ruptured patch formed was 164.6± 17.7 ms (n= 10 cells from 7 patients, mean± s.e.m.) compared to 141.4± 8.5 ms (n= 68 cells from 13 patients, mean± s.e.m.) observed from cells in which the seal formed via perforation. Similarly action potential repolarisation at 90% (APD_{90}) observed from cells in which the seal ruptured during formation was 234.7± 22.6 ms (n= 10 cells from 7 patients, mean± s.e.m.) compared to 213.6± 10.1 ms (n= 68 cells from 13 patients, mean± s.e.m.) observed from cells in which the seal formed via perforations.

4.5.4.3 Lack of effect of method of electrical access to the cell on myocyte refractoriness

There were no significant differences observed in atrial myocyte refractoriness associated with ruptured as compared with perforated patch formation. The mean effective refractory period (ERP) recorded from cells in which the seal ruptured during formation was 223.0± 19.3 ms (n= 10 cells from 7 patients, mean± s.e.m.) compared to 207.3± 8.9 ms (n= 64 cells from 13 patients, mean± s.e.m.) observed from cells in which the seal formed via perforations. The relationship of repolarisation to refractoriness (ERP/APD_{90}) was similar regardless of the method of electrical access to the cell: recordings performed via ruptured patch (NR) demonstrated an ERP/APD_{90} ratio of 0.968± 0.03 (n= 10 cells from 7 patients, mean± s.e.m.) compared to 1.013± 0.04 (n= 64 cells from 13 patients, mean± s.e.m.) from recordings obtained via a perforated patch (NP).

4.5.5 Impact of patient senescence and gender on human atrial isolated myocyte action potentials and refractoriness

4.5.5.1 Lack of effect of patient age (dichotomised) on action potential characteristics

There were no significant differences in the action potential characteristics relating to depolarisation, repolarisation or refractoriness under control conditions between those myocytes isolated from tissue excised from the right atrial appendage of patients aged 75 or over (elderly) as compared with patients aged less than 75 years (young).

4.5.5.2 Impact of patient gender on action potential characteristics

There were no significant differences in the MDP nor V_{max} of phase 0 of the action potential, however a significantly greater amplitude of the action potential upstroke, with similarly greater overshoot that did not reach statistical significance, was observed in atrial myocytes isolated from tissues excised from the RAA of male patients (Figure 4.7 A-D). The mean MDP observed from myocytes emanating from female patients was -83.4± 0.8 mV (n= 15 cells from 4 patients, mean± s.e.m.) compared to -83.7± 0.7 mV (n= 57 cells from 15 patients, mean± s.e.m., Figure 4.7A) from male patients. The maximal rate of depolarization (V_{max}) observed in cells from female patients was 193.9± 10.3 V/s (n= 15 cells from 4 patients, mean± s.e.m.) which was not significantly different from that observed in cells from male patients 208.8± 6.7 V/s (n= 57 cells from 15 patients, mean± s.e.m., Figure 4.7B). The action potential overshoot was similar regardless of patient gender: mean overshoot of 57.5± 2.1 mV (n= 15 cells from 4 patients, mean± s.e.m.) in cells from female patients compared to 61.4± 1.0 mV (n= 57 cells from 15 patients, mean± s.e.m., p= 0.08. Figure 4.7C) in cells from male patients. However the total amplitude of the phase 0 response of the action potential was significantly larger in cells originating from male patients. Mean amplitude of phase 0 response recorded from cells obtained from female patients was 140± 1.6 mV (n= 15 cells from 4 patients, mean± s.e.m.) and was 145.8± 0.9 mV (n= 57 cells from 15 patients, mean± s.e.m., p= 0.006. Figure 4.7D) from cells obtained from male patients.

In contrast, there were no significant differences in the action potential repolarisation nor refractoriness of those atrial myocytes originating from female as compared with male patients.

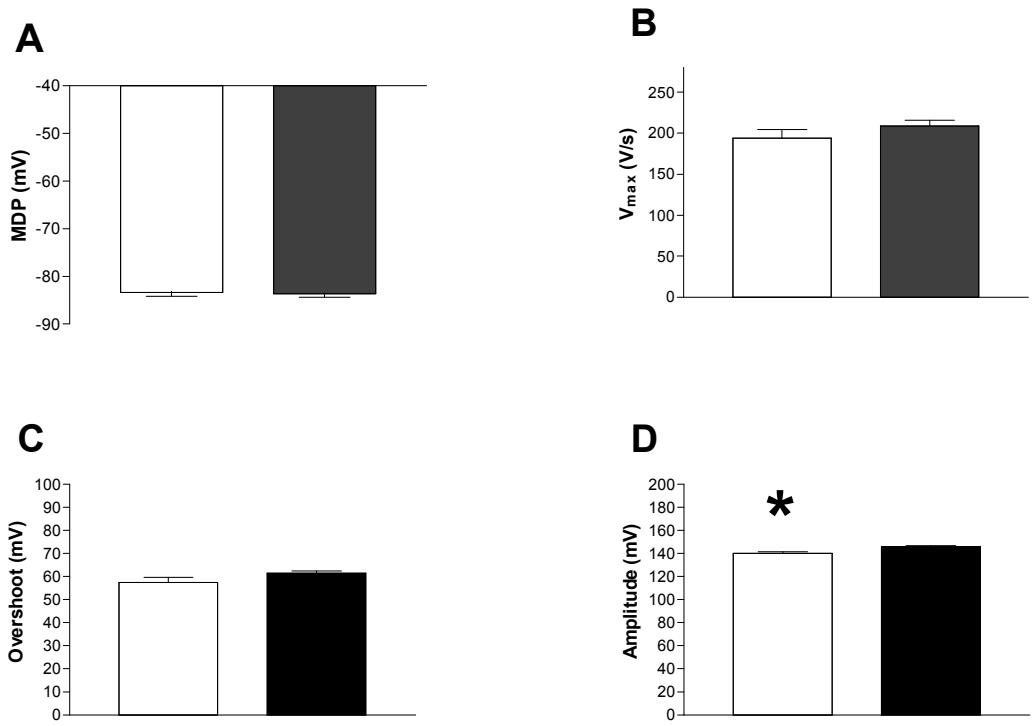


Figure 4.7 Effect of patient gender on resting membrane potential and phase 0 action potential characteristics of human atrial isolated myocytes under control conditions. □ = myocytes obtained from female patients (n= 15 cells from 4 patients) ■ = myocytes obtained from male patients (n= 57 cells from 15 patients). Values are mean± s.e.m., asterisk* indicates p< 0.05.

4.5.6 Impact of chronic pre-operative drug therapy on human atrial isolated myocyte action potentials and refractoriness

4.5.6.1 Lack of effect of chronic pre-operative beta-adrenoceptor blocker therapy on isolated atrial myocyte action potential characteristics

There were no significant differences in the characteristics of depolarisation, repolarisation nor refractoriness under control conditions when myocytes isolated from tissue excised from the right atrial appendage of patients chronically receiving β-B therapy pre-operatively (BBY) were compared to those from patients who were not receiving β-adrenoceptor blocking drug therapy (BBN).

4.5.6.2 Effects of chronic pre-operative calcium channel blocker (CCB) therapy on atrial myocyte action potential characteristics

There were no significant differences in the maximal diastolic potential (MDP) nor of phase 0 of the action potential depolarization characteristics of those myocytes isolated from tissue excised from the right atrial appendage of patients chronically receiving calcium channel blocking drug therapy pre-operatively (CCBY) as compared to those patients who were not receiving calcium channel blocking drug therapy (CCBN) (Figure 4.8 A-D).

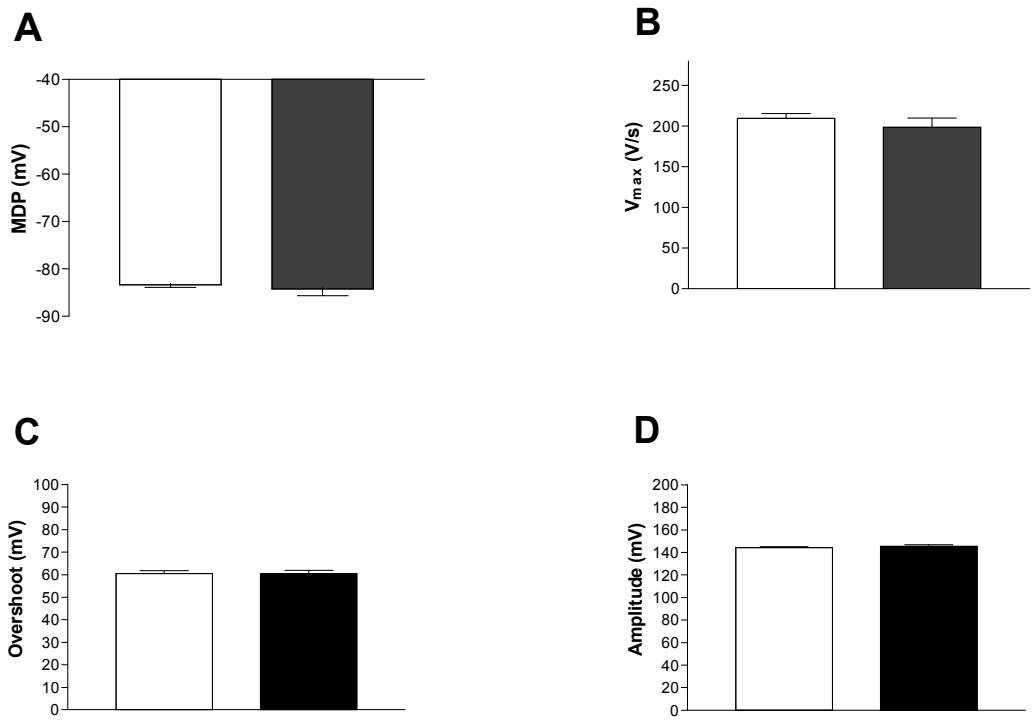


Figure 4.8 Lack of effect of chronic preoperative calcium channel blocking drug therapy on resting membrane potential and phase 0 action potential characteristics of human isolated atrial myocytes under control conditions. □ = myocytes obtained from CCBN patients (n= 47 cells from 14 patients) ■ = myocytes obtained from CCBY patients (n= 25 cells from 5 patients). Values are mean± s.e.m.

In contrast, atrial myocytes isolated from RAA preparations obtained from patients who were chronically receiving CCB therapy pre-operatively demonstrated significantly accelerated action potential repolarisation under control conditions. There were significant differences in early, mid and late action potential repolarisation in comparison to those atrial myocytes originating from patients who

were not chronically receiving CCB pre-operatively (Figure 4.9 A-D). Early repolarisation (APD_{50}) observed from cells from CCBN patients was 25± 4 ms (n= 47 cells from 14 patients, mean± s.e.m.) not significantly different than that observed in CCBY patients, 15.6± 3.2 ms (n= 25 cells from 5 patients, mean± s.e.m., Figure 4.9A).

However a significant difference in early repolarisation was demonstrated at 60% repolarisation, mean (APD_{60}) recorded from cells from CCBN patients was 89.3± 7.5 ms (n= 47 cells from 14 patients, mean± s.e.m.) significantly different than that observed in CCBY patients, 65.2± 8.4 ms (n= 25 cells from 5 patients, mean± s.e.m., p= 0.049. Figure 4.9B). Action potential repolarisation at 75% (APD_{75}) observed from cells from CCBN patients was 162.4± 10.3 ms (n= 47 cells from 14 patients, mean± s.e.m.) significantly different in comparison to 116.9± 11.8 ms (n= 25 cells from 5 patients, mean± s.e.m., p= 0.0078. Figure 4.9C) in cells from CCBY patients.

Similarly this effect was maintained from early and mid- repolarisation to late repolarisation (APD_{90}). Recordings from cells originating from patients who were not receiving CCB drugs pre-operatively APD_{90} was found to be 233± 12 ms (n= 47 cells from 14 patients, mean± s.e.m.) significantly longer than the 188.2± 15.6 ms (n= 25 cells from 5 patients, mean± s.e.m., p= 0.029. Figure 4.9D) observed from cells obtained from patients who were receiving CCB drugs pre-operatively.

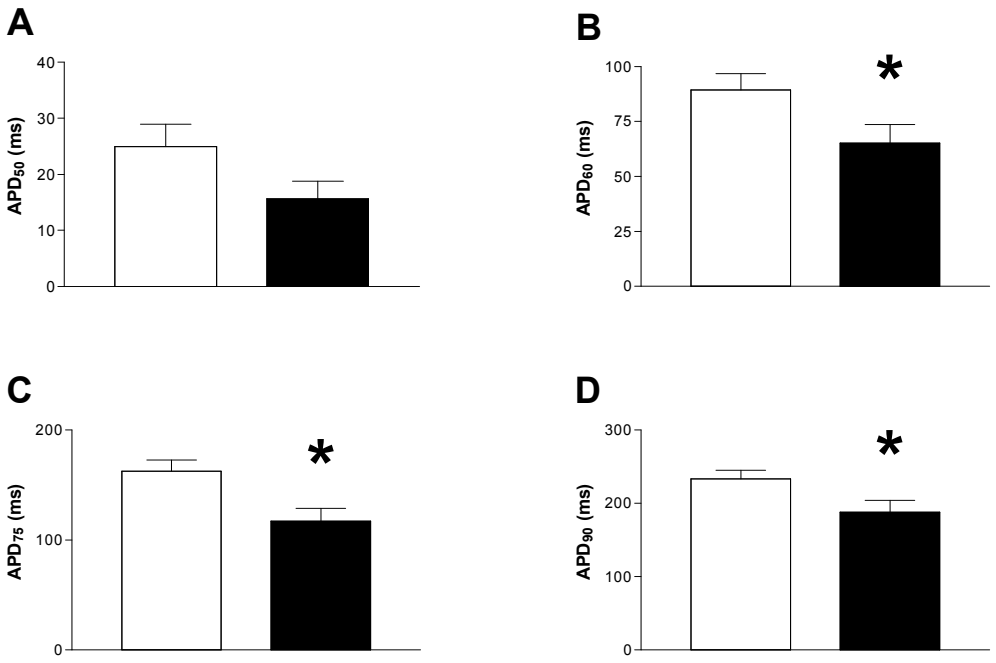


Figure 4.9 Effect of chronic preoperative calcium channel blocking drug therapy on human atrial isolated myocyte action potential repolarisation under control conditions. □ = myocytes obtained from CCBN patients (n= 47 cells from 14 patients) ■ = myocytes obtained from CCBY patients (n= 25 cells from 5 patients). Values are mean± s.e.m., asterisk* indicates $p< 0.05$.

There were no significant differences observed in single cell refractoriness in atrial myocytes isolated from tissue preparations obtained from patients chronically receiving CCB therapy pre-operatively (CCBY) as compared to those patients who were not (CCBN) (Figure 4.10 A and B). The mean ERP recorded from cells obtained from CCBN patients was 214.5± 10 ms (n= 47 cells from 14 patients, mean± s.e.m.) compared to 200.4± 14.9 ms (n= 25 cells from 5 patients, mean± s.e.m., Figure 4.10A) observed from cells obtained from CCBY patients. The relationship of repolarisation to refractoriness (ERP/APD_{90}) was similar regardless of CCB status pre-operatively: recordings made from cells obtained from CCBN patients demonstrated an ERP/APD_{90} ratio of 0.97± 0.02 (n= 47 cells from 14 patients, mean± s.e.m.) compared to 1.076± 0.08 (n= 25 cells from 5 patients, mean± s.e.m., Figure 4.10B) from recordings of cells obtained from CCBY patients.

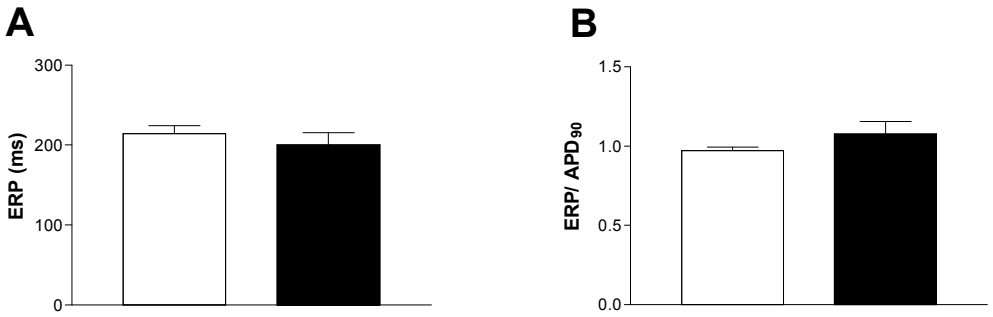


Figure 4.10 Lack of effect of chronic preoperative calcium channel blocker therapy on human atrial isolated myocyte refractoriness under control conditions. □ = myocytes obtained from CCBN patients (n= 47 cells from 14 patients) ■ = myocytes obtained from CCBY patients (n= 25 cells from 5 patients). Values are mean± s.e.m.

4.5.6.3 Impact of chronic pre-operative Angiotensin Converting Enzyme Inhibitor (ACE-I) therapy on atrial myocyte action potential characteristics

There were no significant differences in the MDP, amplitude nor overshoot of the action potential upstroke, however a significantly greater V_{max} of phase 0 of the action potential was observed in atrial myocytes isolated from RAA preparations obtained from patients chronically receiving ACE-I therapy (ACE-IY) compared to myocytes from those patients who were not (ACE-IN) (Figure 4.11 A-D).

The mean MDP observed from myocytes obtained from ACE-IN patients was -83± 1 mV (n= 24 cells from 6 patients, mean± s.e.m.) compared to -83.9± 0.8 mV (n= 48 cells from 13 patients, mean± s.e.m., Figure 4.11A) from ACE-IY patients. The maximal rate of depolarization (V_{max}) observed in cells from ACE-IN patients was 187.6± 9.6 V/s (n= 24 cells from 6 patients, mean± s.e.m.) which was significantly different from that observed in cells from ACE-IY patients 214.7± 6.9 V/s (n= 48 cells from 13 patients, mean± s.e.m., p= 0.025. Figure 4.11B). Neither the action potential overshoot nor overall phase 0 amplitude were associated with pre-operative ACE-I therapy induced effects: the mean overshoot in cells from ACE-IN patients was 59.5± 2.7 mV (n= 24 cells from 6 patients, mean± s.e.m.) compared

to 60.9± 0.9 mV (n= 48 cells from 13 patients, mean± s.e.m., Figure 4.11C) in cells from ACE-IY patients. The total amplitude of the phase 0 response of the action potential recorded from cells obtained from ACE-IN patients was 142.9± 1.6 mV (n= 24 cells from 6 patients, mean± s.e.m.) and was 145.4± 1.0 mV (n= 48 cells from 13 patients, mean± s.e.m., Figure 4.11D) from cells obtained from ACE-IY patients.

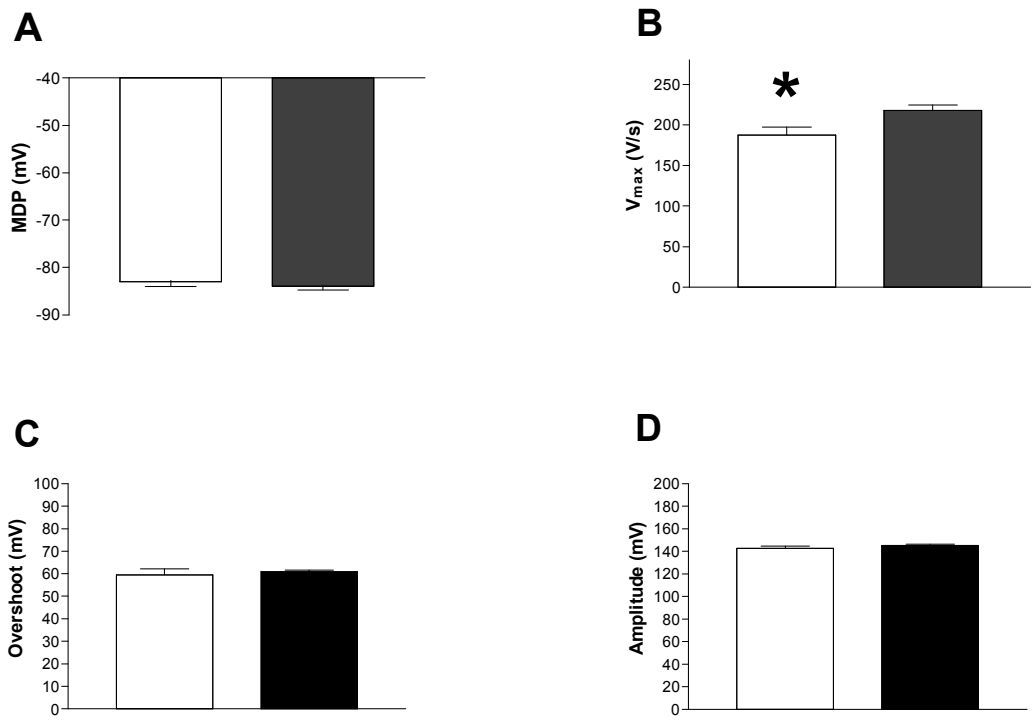


Figure 4.11 Effect of chronic preoperative angiotensin converting enzyme inhibitor (ACE-I) therapy on resting membrane potential and phase 0 action potential characteristics of human atrial isolated myocytes under control conditions. □ = myocytes obtained from ACE-IN patients (n= 24 cells from 6 patients) ■ = myocytes obtained from ACE-IY patients (n= 48 cells from 13 patients). Values are mean± s.e.m., asterisk* indicates p< 0.05.

There were no significant differences observed in action potential repolarisation nor refractoriness from single atrial myocytes isolated from tissue preparations obtained from patients chronically receiving ACE-I therapy pre-operatively (ACE-IY) as compared to those patients who were not (ACE-IN).

4.5.7 Impact of patient comorbidity on human atrial isolated myocyte action potential characteristics

4.5.7.1 Impact of pre-operative left ventricular systolic dysfunction (LVSD) on atrial myocyte action potential characteristics

Atrial myocytes isolated from RAA tissue preparations obtained from patients with LVSD (LVSDY) were significantly more polarized in the quiescent state under control conditions as compared to recordings made from atrial myocytes obtained from patients diagnosed as free from LVSD (LVSDN) pre-operatively (Figure 4.12A). There were, however, no significant differences detected in the observed amplitude nor the overshoot of the action potential upstroke, nor in V_{max} of phase 0 of the action potential (Figure 4.12 B-D). The mean MDP observed from myocytes obtained from LVSDN patients was -82.1± 0.7 mV (n= 35 cells from 10 patients, mean± s.e.m.) significantly different as compared to -84.8± 0.9 mV (n= 43 cells from 10 patients, mean± s.e.m., p= 0.025. Figure 4.12A) from LVSDY patients. The maximal rate of depolarization (V_{max}) observed in cells from LVSDN patients was 208.5± 9.4 V/s (n= 35 cells from 10 patients, mean± s.e.m.) which was not significantly different from that observed in cells from LVSDY patients 209± 6.9 V/s (n= 43 cells from 10 patients, mean± s.e.m. Figure 4.12B). Neither the action potential overshoot nor overall phase 0 amplitude were associated with pre-operative LVSD status: the mean overshoot in cells from LVSDN patients was 60.2± 1.1 mV (n= 35 cells from 10 patients, mean± s.e.m.) compared to 61.4± 1.5 mV (n= 43 cells from 10 patients, mean± s.e.m., Figure 4.12C) in cells from LVSDY patients. The total amplitude of the phase 0 response of the action potential recorded from cells obtained from LVSDN patients was 145± 1.1 mV (n= 35 cells from 10 patients, mean± s.e.m.) and was 144± 1.5 mV (n= 43 cells from 10 patients, mean± s.e.m., Figure 4.12D) from cells obtained from LVSDY patients.

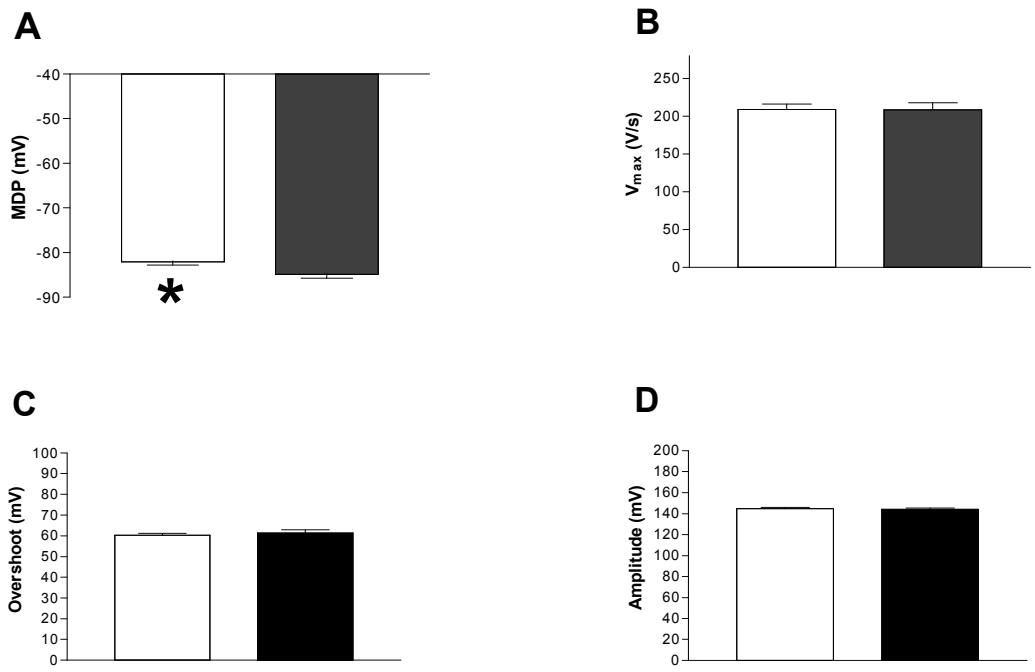


Figure 4.12 Effect of pre-operative left ventricular systolic dysfunction (LVSD) on resting membrane potential and phase 0 action potential characteristics of human atrial isolated myocytes under control conditions. □ = myocytes obtained from patients without LVSD (n= 35 cells from 10 patients) ■ = myocytes obtained from patients with LVSD (n= 43 cells from 10 patients). Values are mean± s.e.m., asterisk* indicates $p< 0.05$.

There were no significant differences observed in action potential repolarisation nor refractoriness from single atrial myocytes isolated from tissue preparations obtained from patients suffering pre-operative LVSD as compared to those patients who were not (Figure 4.13 A-C and 4.14 A and B).

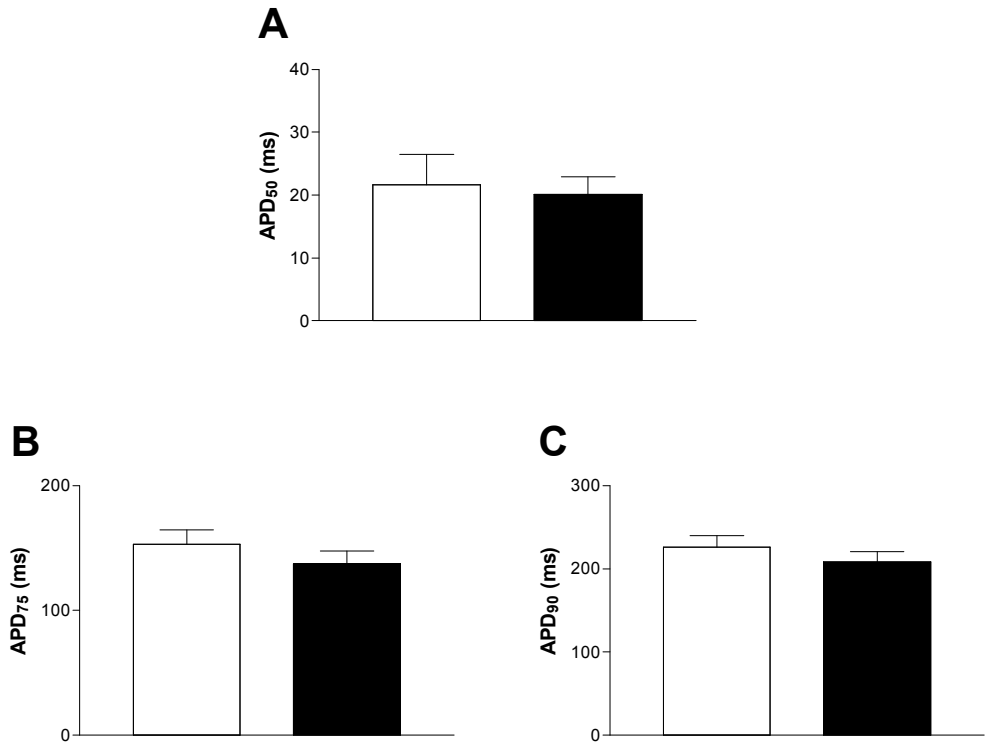


Figure 4.13 Lack of effect of pre-operative left ventricular systolic dysfunction (LVSD) on human atrial isolated myocyte action potential repolarisation under control conditions. □ = myocytes obtained from patients without LVSD (n= 35 cells from 10 patients) ■ = myocytes obtained from patients with LVSD (n= 43 cells from 10 patients). Values are mean± s.e.m.

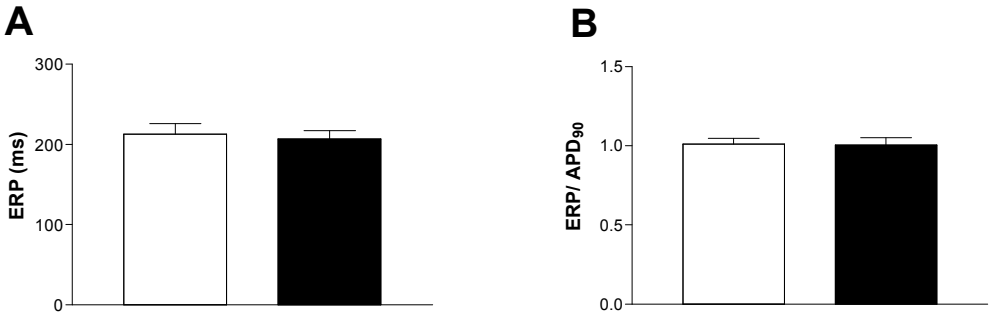


Figure 4.14 Lack of effect of pre-operative left ventricular systolic dysfunction (LVSD) on human atrial isolated myocyte refractoriness under control conditions. □ = myocytes obtained from patients without LVSD (n= 35 cells from 10 patients) ■ = myocytes obtained from patients with LVSD (n= 43 cells from 10 patients). Values are mean± s.e.m.

4.5.7.2 Lack of effect of prior myocardial infarction (MI) on atrial myocyte action potential characteristics

There were no significant differences in the characteristics of depolarisation, repolarisation nor refractoriness observed in recordings made from those myocytes isolated from tissue excised from the right atrial appendage of patients

who had suffered a myocardial infarction (MI) pre-operatively (MIY) as compared to those patients who had not suffered a prior MI (MIN).

4.5.8 Effect of 0.05 μM isoproterenol alone on human atrial isolated myocyte action potentials and refractoriness

4.5.8.1 Effect of 0.05 μM ISO on human atrial isolated myocyte action potential characteristics relating to depolarisation

Isoproterenol, at 0.05 μM, had no significant effect on action potential upstroke, amplitude nor on phase one overshoot of the action potential but was associated with a small but statistically significant depolarisation of the myocyte during diastole as illustrated in Figure 4.15 and 4.16.

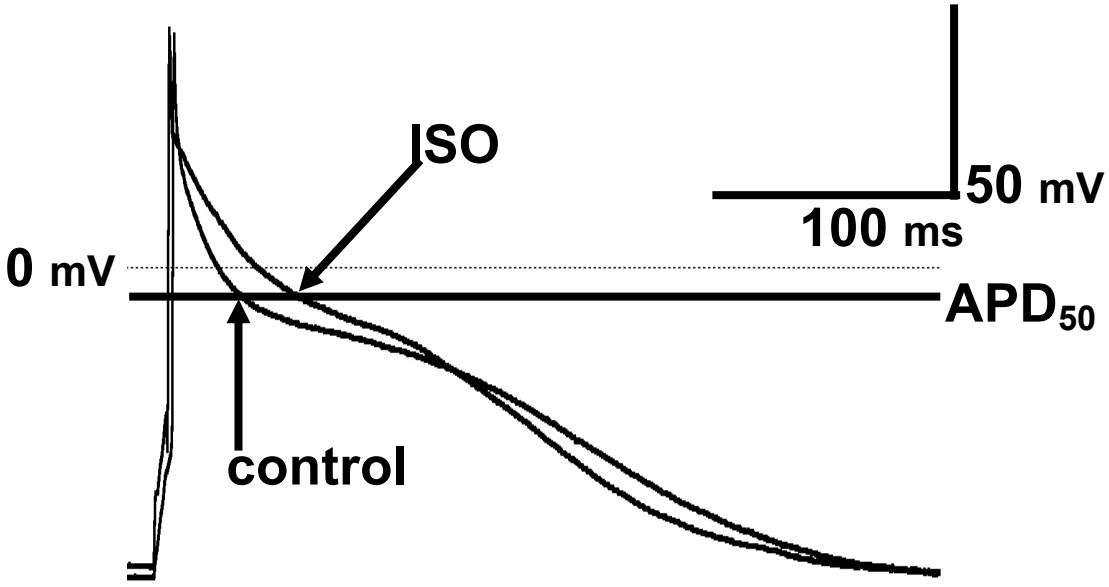


Figure 4.15 A representative example of the effect of acute superfusion with 0.05 μM Isoproterenol on a human atrial isolated myocyte action potential waveform.

The mean MDP under control conditions was -83.6± 0.7 mV whereas following acute superfusion with 0.05 μM ISO the mean MDP was observed to be significantly different at -78.9± 1.3 mV (n= 22 cells from 12 patients, mean± s.e.m., paired data, p< 0.05, Figure 4.16A). The maximal rate of depolarization (V_{max}), however, was not significantly different following acute superfusion with ISO at 0.05 μM (control V_{max} 194.1± 9.1 V/s vs 196.9± 11.3 V/s following 0.05 μM ISO, n= 22 cells from 12 patients, mean± s.e.m., paired data, Figure 4.16B). The action potential overshoot was also similar regardless of the presence of 0.05 μM ISO in the superfusate (55.6± 1.5 mV vs 57.3± 1.6 mV (n= 22 cells from 12 patients, mean± s.e.m., paired data Figure 4.16C). Similarly the total amplitude of the phase 1 response of the action potential did not significantly differ during acute superfusion with ISO at 0.05 μM: mean amplitude of phase 1 response recorded from cells under control conditions was 140.6± 1.6 mV compared to 136.6± 2.0 mV during exposure to ISO (n= 22 cells from 12 patients, mean± s.e.m., Figure 4.16D).

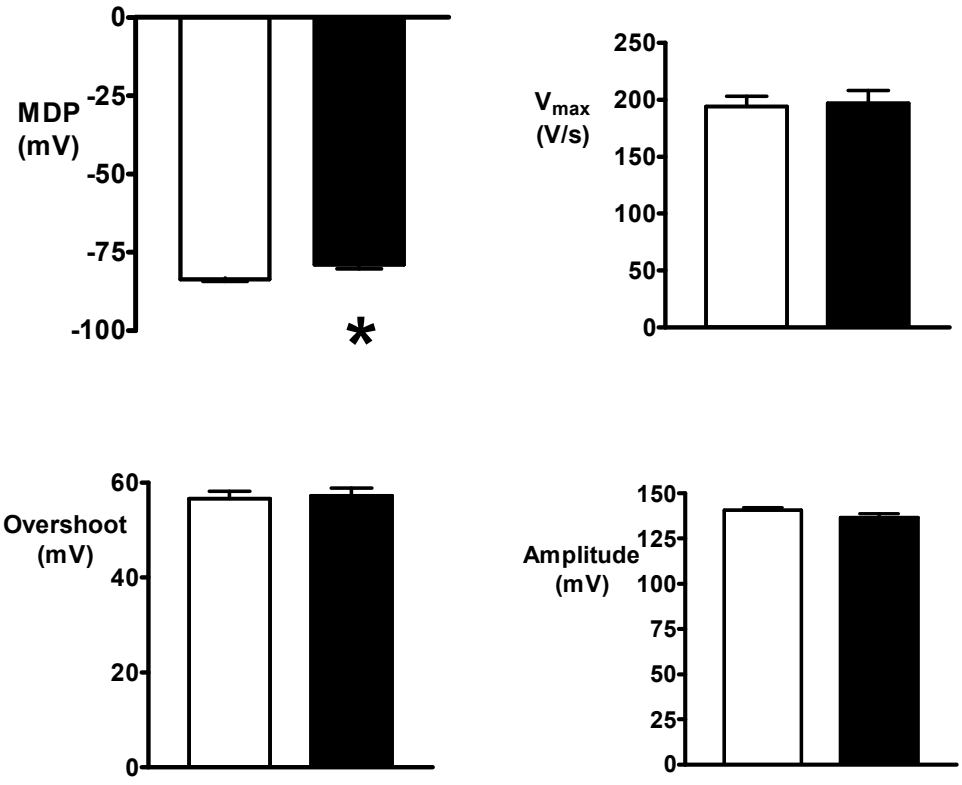


Figure 4.16 Effect of acute superfusion with 0.05 μM Isoproterenol on resting membrane potential and phase 1 action potential characteristics of human atrial isolated myocytes. □ = control ■ = 0.05 μM Isoproterenol (ISO) (n= 22 cells from 12 patients, paired data). Values are mean± s.e.m., asterisk (*) p< 0.05.

4.5.8.2 Effect of ISO on human atrial isolated myocyte action potential repolarisation

Isoproterenol, at 0.05 μM, significantly prolonged the early and mid repolarisation of human atrial myocyte action potentials resulting in elevation of the action potential plateau, but had no such effect on later repolarisation or effective refractory period (Figures 4.17 and 4.19). The APD_{50} significantly increased from 28± 5 ms under control conditions to 54± 10 ms following the application of ISO, an increase of 93% (n= 22 cells from 12 patients, p< 0.05 Figure 4.17). Neither the APD_{75} (control 154± 13 ms vs 163± 21 ms post ISO, n= 22 cells from 12 patients Figure 4.17) nor the APD_{90} (control 220± 14 ms vs 204± 16 ms post ISO, n= 22 cells from 12 patients Figure 4.17) was significantly affected.

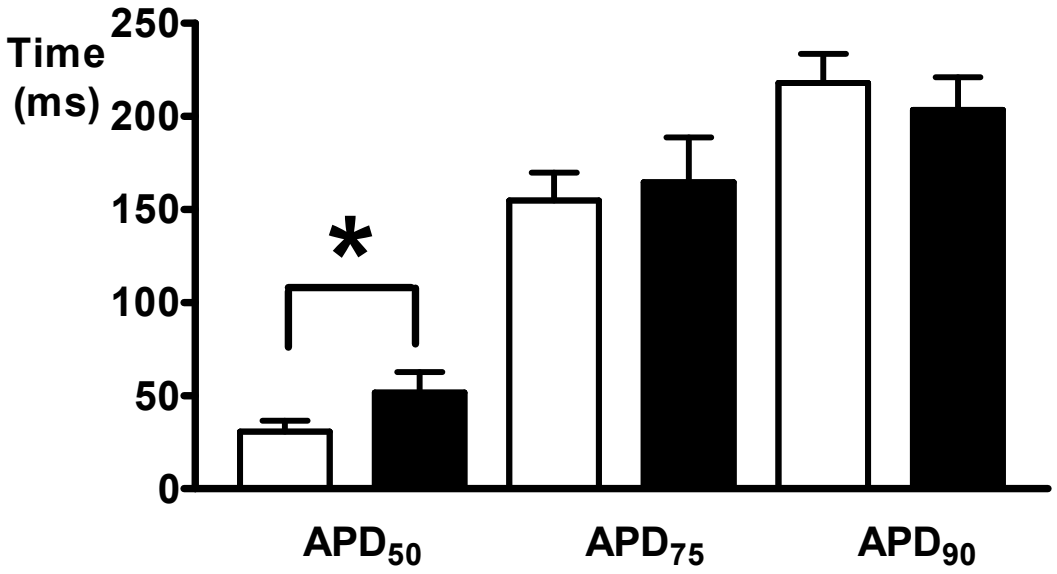


Figure 4.17 Effect of acute superfusion with 0.05 μM Isoproterenol on action potential repolarisation characteristics of human atrial isolated myocytes. □ = control ■ = 0.05 μM Isoproterenol (ISO) (n= 22 cells from 12 patients, paired data). Values are mean± s.e.m., asterisk (*) p< 0.05.

4.5.8.3 Effect of ISO on human atrial isolated myocyte refractoriness

An example of the effect of acute superfusion with 0.05μM ISO on human isolated atrial myocyte refractoriness can be seen in Figure 4.20 and mean data in Figure 4.19. Although 0.05μM ISO did not have a significant effect on the ERP directly (control ERP 208.9± 16.7 ms vs ISO ERP 227.9± 28.8 ms, n= 19 cells from 10 patients Figure 4.19), the combination of this non significant increase in ERP and the non significant decrease in the APD_{90} (see section 4.5.8.2) resulted in a small but statistically significant increase in the ERP/ APD_{90} ratio (control ERP/APD_{90} 0.976± 0.02 vs ISO ERP/APD_{90} 1.04± 0.03 ms, n= 19 cells from 10 patients, p< 0.05 Figure 4.19).

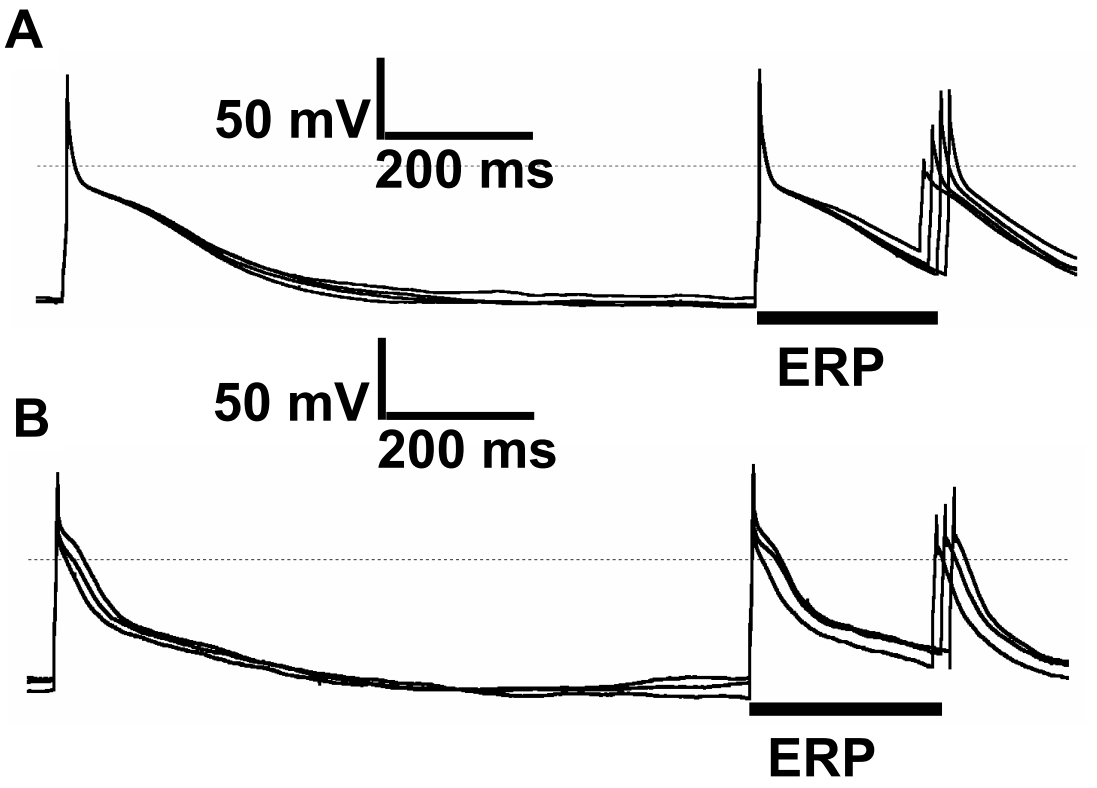


Figure 4.18 Representative example of the effect of acute superfusion with 0.05 µM Isoproterenol on human atrial isolated myocyte action potential waveforms, A) under control conditions and B) during acute superfusion with ISO.

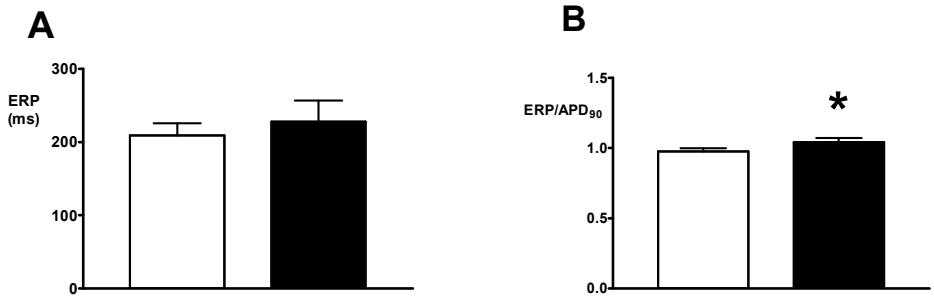


Figure 4.19 Effect of acute superfusion with 0.05 µM Isoproterenol on human atrial isolated myocyte refractoriness. □ = control ■ = 0.05 µM Isoproterenol (ISO) (n= 19 cells from 10 patients, paired data). Values are mean± s.e.m., asterisk (*) p< 0.05.

4.5.8.4 Effect of ISO to induce spontaneous depolarisations

During the repetitive stimulation protocol designed to evaluate the effective refractory period following superfusion with ISO, but not under control conditions, low amplitude sub-threshold spontaneous depolarisations would frequently

interrupt the stimulation train. These 'spontaneous depolarisations' (SD) most frequently occurred during phase 3 and 4 of the action potential and only occasionally reached threshold (see Figure 4.20). The occurrence of SDs was significantly associated with exposure to ISO and was not observed under control conditions (control SD 0% vs ISO SD 64%, n= 11 cells, 3 patients, $\chi 2$ test, $p< 0.05$).

4.5.9 Effect of 10 nM endothelin-1 alone on human atrial isolated myocyte action potentials and refractoriness

4.5.9.1 Effect of ET-1 alone on human atrial myocyte action potential characteristics related to depolarisation

Acute superfusion of human atrial myocytes with 10 nM Endothelin-1 had no apparent effect on the MDP, amplitude nor on phase one overshoot of the action potential but was associated with a significant reduction in action potential upstroke velocity (Figure 4.21). Under control conditions the MDP was -84.8± 1.5 mV and following acute superfusion with 10 nM ET-1 was observed to be -83.6± 2 mV (n= 21 cells from 5 patients, mean± s.e.m., paired data, Figure 4.21A). The maximal rate of depolarization (V_{max}), however, was significantly reduced following exposure to 10 nM ET-1 (control V_{max} 211± 12.3 V/s vs 165± 11.9 V/s following 10 nM ET-1, n= 21 cells from 5 patients, mean± s.e.m., paired data, Figure 4.21B). The action potential overshoot was similar regardless of the presence of ET-1 in the superfusate (61.9± 1.7 mV vs 55.9± 2.9 mV (n= 21 cells from 5 patients, mean± s.e.m., paired data, Figure 4.21C). The total amplitude of the phase 1 response of the action potential did not significantly differ during acute superfusion with 10 nM ET-1: mean amplitude of phase 1 response recorded from cells under control conditions was 146.9± 1.6 mV compared to 139± 4.3 mV during exposure to ET-1 (n= 21 cells from 5 patients, mean± s.e.m., paired data, Figure 4.21D).

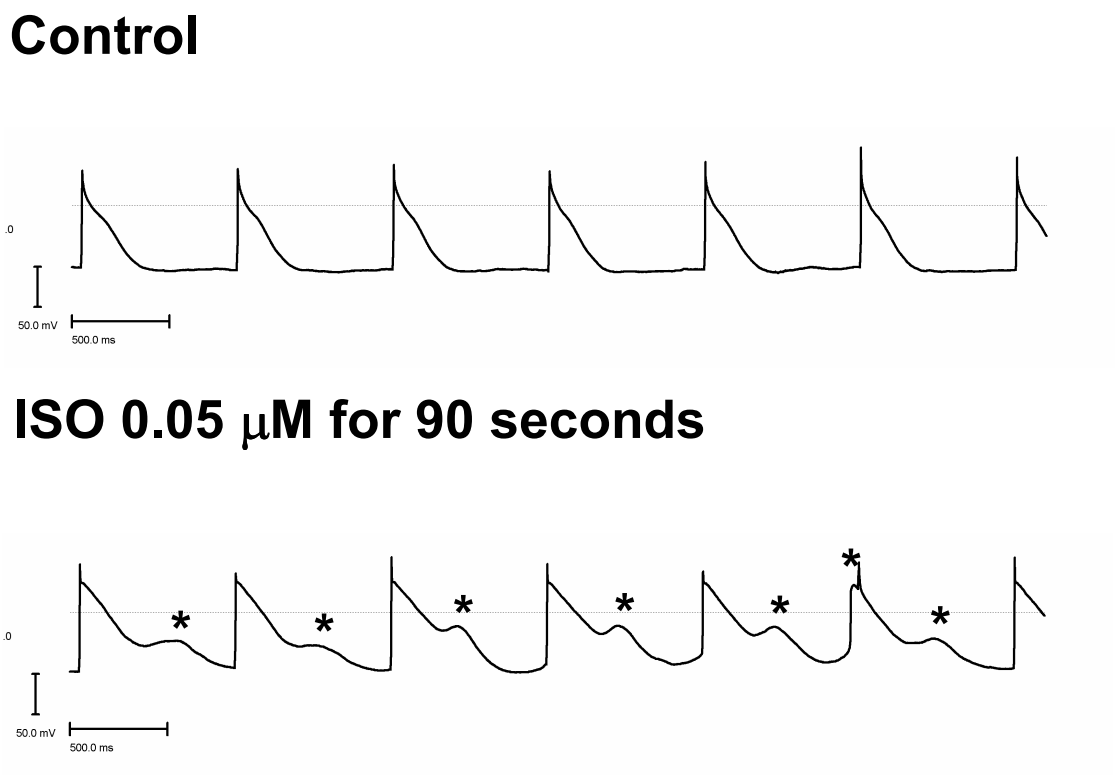


Figure 4.20 A representative example of the effect of acute superfusion with 0.05 μM Isoproterenol on a human atrial isolated myocyte action potentials stimulated during a repetitive stimulation protocol demonstrating phase 3 spontaneous depolarisations (*).

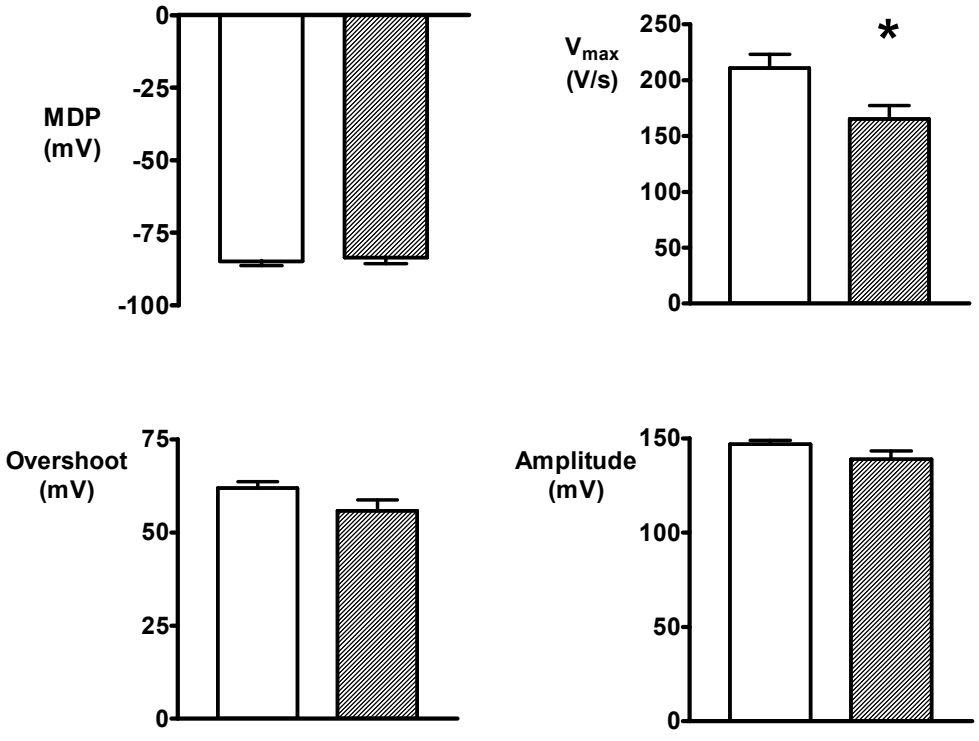


Figure 4.21 Effect of acute superfusion with 10 nM ET-1 on resting membrane potential and phase 1 action potential characteristics of human atrial isolated myocytes. □ = control ▨ = 10 nM ET-1 (n= 21 cells from 5 patients, paired data). Values are mean± s.e.m., asterisk (*) p< 0.05.

4.5.9.2 Effect of ET-1 alone on action potential repolarisation

The acute superfusion of human atrial myocytes with 10 nM ET-1 had no discernible effect on action potential repolarisation (Figures 4.22 and 4.24). The APD_{50} was similar under control conditions compared to during superfusion with ET-1 (12.8± 1.9 ms vs 13.8± 1.8 n= 21 cells from 5 patients, Figures 4.22 and 4.24). Neither the APD_{75} (control 132.4± 13.1 ms vs 141.8± 13.2 ms post ET-1, n= 21 cells from 5 patients, Figure 4.22) nor the APD_{90} (control 210.1± 17.1 ms vs 226.2± 19.7 ms post ET-1, n= 21 cells from 5 patients) was significantly different during exposure to 10 nM ET-1 (Figure 4.22).

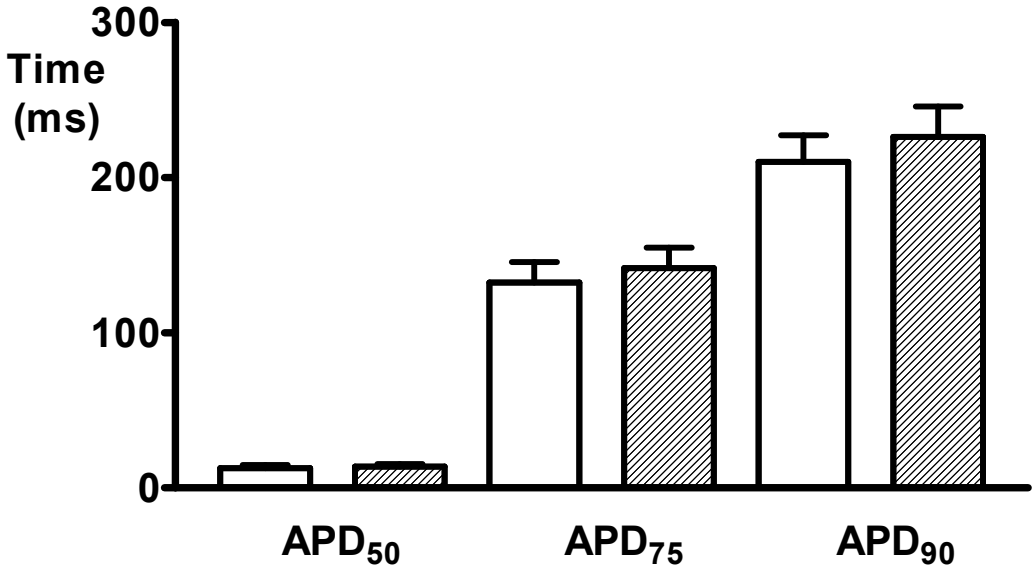


Figure 4.22 Effect of acute superfusion with 10 nM ET-1 on action potential repolarisation characteristics of human atrial isolated myocytes. □ = control ▨ = 10 nM ET-1 (n= 21 cells from 5 patients, paired data). Values are mean± s.e.m.

4.5.9.3 Effect of ET-1 alone on effective refractory period

An example of the effect of acute superfusion with 10 nM ET-1 on human refractoriness can be seen in Figure 4.23 and mean data in Figures 4.23 & 4.24. Superfusion of myocytes with 10 nM ET-1 did not have a significant effect on the ERP (control ERP 190.6± 14.9 ms vs ET-1 ERP 207.5± 15.5 ms, n= 16 cells from 5 patients Figures 4.23 and 4.24), nor the ERP/ APD_{90} ratio (control ERP/APD_{90}

0.903± 0.03 vs ET-1 ERP/APD_{90} 0.957± 0.04 ms, n= 16 cells from 5 patients, Figure 4.23).

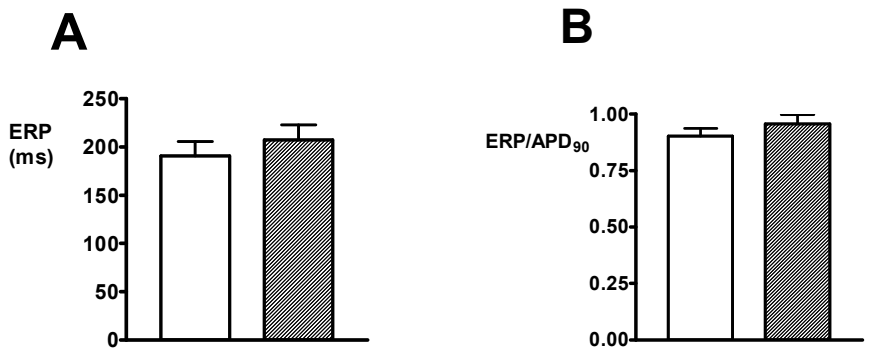


Figure 4.23 Effect of acute superfusion with 10 nM ET-1 on human atrial isolated myocyte refractoriness. □ = control ▨ = 10 nM ET-1 (n= 16 cells from 5 patients, paired data). Values are mean± s.e.m.

4.5.9.4 Effect of ET-1 alone on spontaneous depolarisations

Acute superfusion with 10 nM ET-1 alone was not significantly associated with SD occurrence (control SD 0% vs ET-1 SD 15%, n= 13 cells, 5 patients, $\chi 2$ test, p= ns).

4.5.10 Effect of 0.05 μM ISO alone and in combination (co-application) with 10 nM ET-1 on human isolated atrial myocyte action potentials and refractoriness

4.5.10.1 Effect of ET-1 on adrenergically prestimulated action potential characteristics relating to depolarisation

Acute superfusion of human atrial myocytes with 0.05 μM Isoproterenol followed by the addition of 10 nM Endothelin-1 to the superfusate (co-application) after 5 minutes had no apparent effect on the MDP, action potential upstroke velocity, amplitude nor on phase one overshoot of the action potential (Figure 4.25). Under control conditions the MDP was -83.3± 0.8 mV, following acute superfusion with 0.05 μM ISO the MDP was -79.8± 1.1 mV and was -79.5± 1.3 mV following the

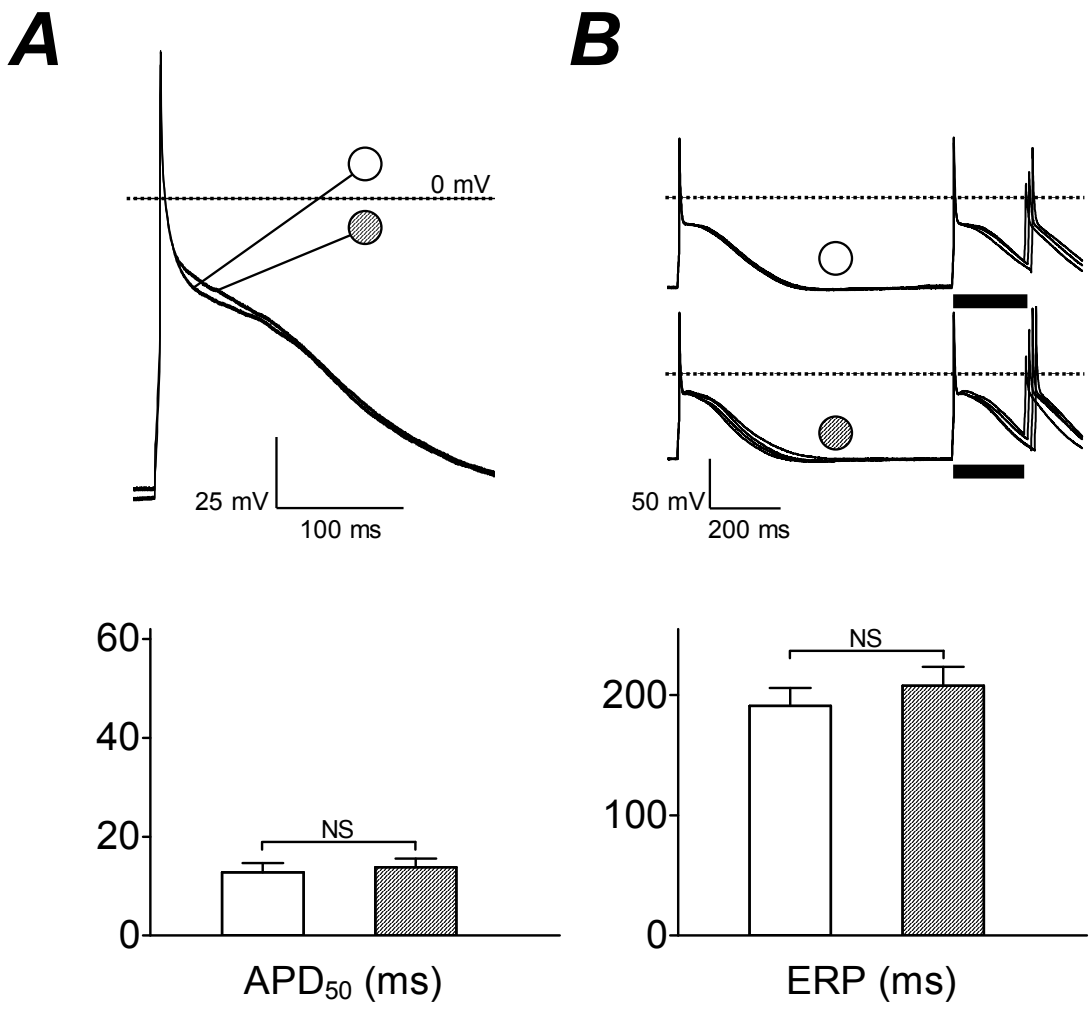


Figure 4.24 Lack of effect of ET-1 on basal action potential repolarisation and ERP. A, Action potential waveform and mean APD_{50}, and B, Restitution and mean ERP, in absence of a drug (○) and following 10 nM ET-1 (▨). Upper panels: superimposed responses to (A) 7th conditioning pulse and (B) in a different cell, to the 7th and 8th conditioning pulses followed by premature test pulses. Horizontal bars=ERP. Lower panels: paired means of APD_{50} (21 cells, 5 patients) and ERP (16 cells, 5 patients). Values mean ± s.e.m. Figure taken from Redpath et al, JMCC 2006; 40: 717-724.

addition of 10 nM ET-1 to the superfusate (n= 15 cells from 10 patients, mean± s.e.m., paired data, Figure 4.25A). The maximal rate of depolarization (V_{max}) did not significantly change following superfusion with ISO, nor with ISO and ET-1 in combination (control V_{max} 191.4± 11.3 V/s, ISO V_{max} 203.4± 9.6 V/s and ISO/ET-1 V_{max} 182.9± 15.6 V/s, n= 15 cells from 10 patients, mean± s.e.m., paired data, Figure 4.25B). The action potential overshoot was similar regardless of the presence of ISO or subsequently simultaneous ISO and ET-1 in the superfusate (control overshoot 56.9± 1.8 mV vs ISO 59.1± 1.5 mV vs ISO/ET-1 59.5± 2.5 mV, n= 15 cells from 10 patients, mean± s.e.m., paired data, Figure 4.25C). The total

amplitude of the phase 1 response of the action potential did not significantly differ following superfusion with 0.05 μM ISO alone or in combination with 10 nM ET-1: mean amplitude of phase 1 response recorded from cells under control conditions was 140.5± 1.9 mV compared to 139.1± 1.1 mV during exposure to 0.05 μM ISO alone and was 138.9± 2.3 mV during superfusion with ISO and ET-1 (n= 15 cells from 10 patients, mean± s.e.m., paired data, Figure 4.25D).

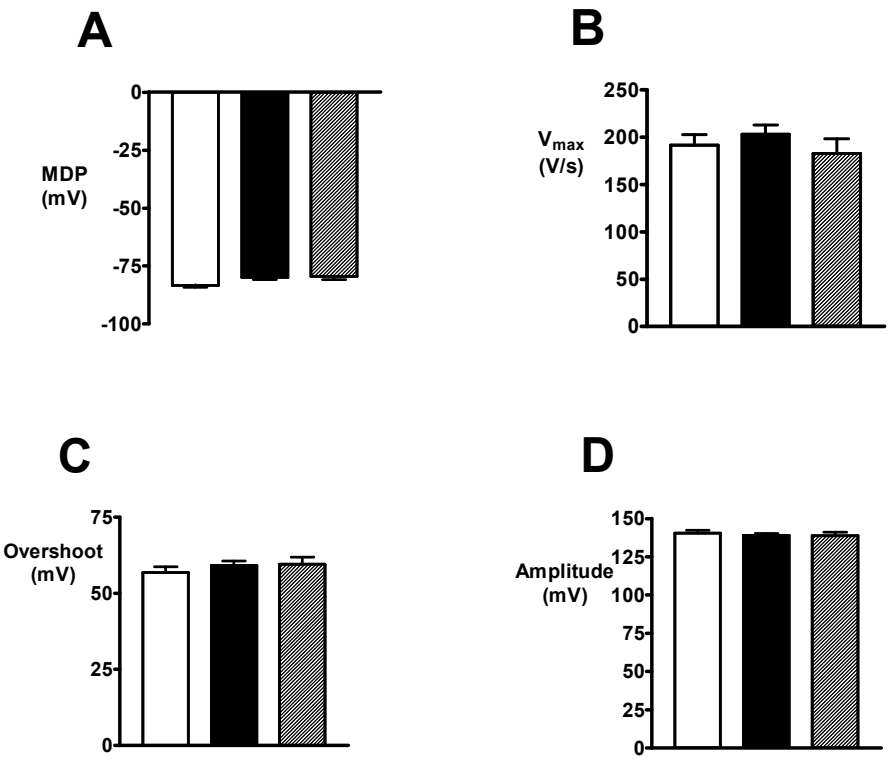


Figure 4.25 Effect of acute superfusion with 0.05 μM ISO alone and then 0.05 μM ISO in combination with 10 nM ET-1 on resting membrane potential and phase 1 action potential characteristics of human atrial isolated myocytes. □ = control ■ = 0.05 μM ISO ▨ = 0.05 μM ISO and 10 nM ET-1 co-application (n= 15 cells from 10 patients, paired data). Values are mean± s.e.m.

4.5.10.2 Effect of ET-1 on adrenergically prestimulated action potential repolarisation (APD)

As described in sections 4.5.8.2 and 4.5.8.3, 0.05 μM Isoproterenol significantly prolonged the early and mid repolarisation of human atrial myocyte action potentials resulting in elevation of the action potential plateau, but had no such effect on later repolarisation or effective refractory period, the “adrenergic effect” on action potentials (Figures 4.17 to 4.19). In cells in which the adrenergic effect of

ISO alone was recorded prior to addition of 10 nM ET-1 to the superfusate the APD_{50} significantly increased from 30± 6.3 ms under control conditions to 45.7± 6.9 ms following the application of ISO, an increase of 52% (n= 15 cells from 10 patients, paired data, p< 0.05, Figure 4.26) was then significantly reversed to 19.8± 3.3 ms during superfusion of myocytes with 0.05 μM ISO and 10 nM ET-1 in combination (n= 15 cells from 10 patients, paired data, p< 0.05, Figure 4.26).

Neither the APD_{75} (control 144.9± 16 ms vs 137.3± 14.9 ms post ISO vs 111± 13 ms post ISO and ET-1, n= 15 cells from 10 patients) nor the APD_{90} (control 210.3± 17.3 ms vs 187.9± 16.1 ms post ISO vs 173.5± 13.2 ms post ISO and ET-1, n= 15 cells from 10 patients) was significantly affected either by ISO alone nor ISO in combination with ET-1 (Figure 4.26).

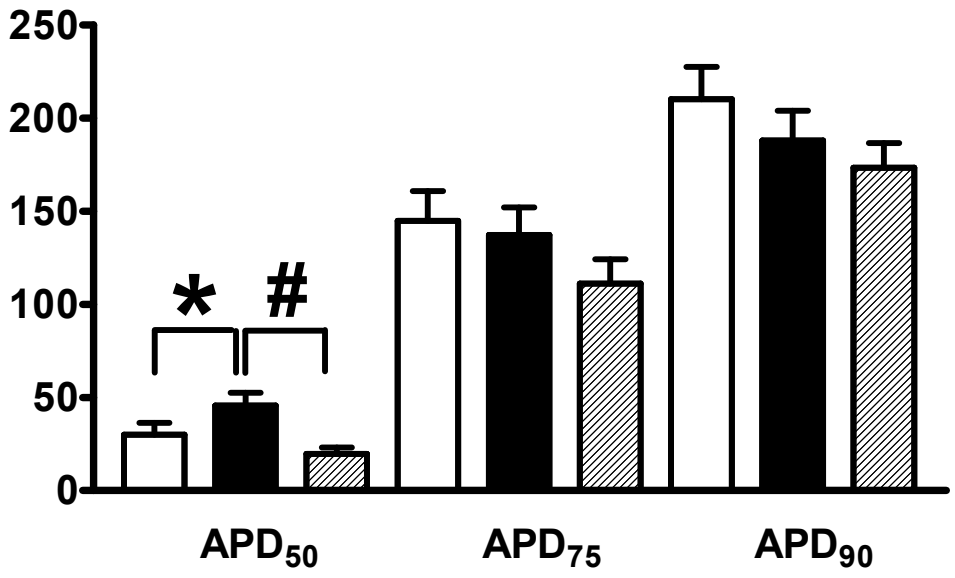


Figure 4.26 Effect of acute superfusion with 0.05 μM ISO alone and then 0.05 μM ISO in combination with 10 nM ET-1 on action potential repolarisation of human atrial isolated myocytes. □ = control ■ = 0.05 μM ISO ▨ = 0.05 μM ISO and 10 nM ET-1 co-application (n= 15 cells from 10 patients, paired data). Values are mean± s.e.m., asterisk (*) and hash (#) indicate p< 0.05.
Figure taken from Redpath et al, JMCC 2006; 40: 717-724.

4.5.10.3 Effect of ET-1 on adrenergically prestimulated action potential effective refractory period

An example of the effects of acute superfusion with 0.05 μM ISO alone and subsequent co-application of 0.05 μM ISO with 10 nM ET-1 on human refractoriness can be seen in Figure 4.27 and mean data in Figures 4.28 & 4.29. Superfusion of myocytes with ISO alone and in combination with 10 nM ET-1 did not have a significant effect on cellular ERP (control ERP 204± 19 ms vs ISO ERP 198.7± 15.9 vs ISO/ET-1 ERP 180± 15 ms, n= 15 cells from 10 patients Figures 4.27 and 4.28), nor the ERP/ APD_{90} ratio (control ERP/APD_{90} 0.962± 0.03 vs ISO ERP 1.078± 0.04 vs ISO/ET-1 ERP/APD_{90} 1.04± 0.04 ms, n= 15 cells from 10 patients, Figure 4.27).

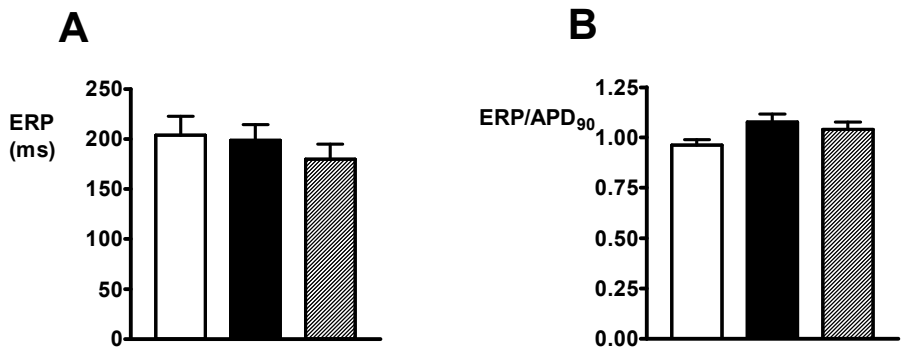


Figure 4.27 Effect of acute superfusion with 0.05 μM ISO alone and then 0.05 μM ISO in combination with 10 nM ET-1 on human atrial isolated myocyte refractoriness. □ = control ■ = 0.05 μM ISO ▨ = 0.05 μM ISO and 10 nM ET-1 co-application (n= 15 cells from 10 patients, paired data). Values are mean± s.e.m.

4.5.10.4 Effect of ET-1 on adrenergically prestimulated spontaneous depolarisations

During repetitive stimulation protocol testing to evaluate the effective refractory period following superfusion with ISO, but not under control conditions, low amplitude sub-threshold phase 3 and 4 spontaneous depolarisations frequently occurred, as described in section 4.5.8.4 (Figures 4.20 and 4.29). These SDs did

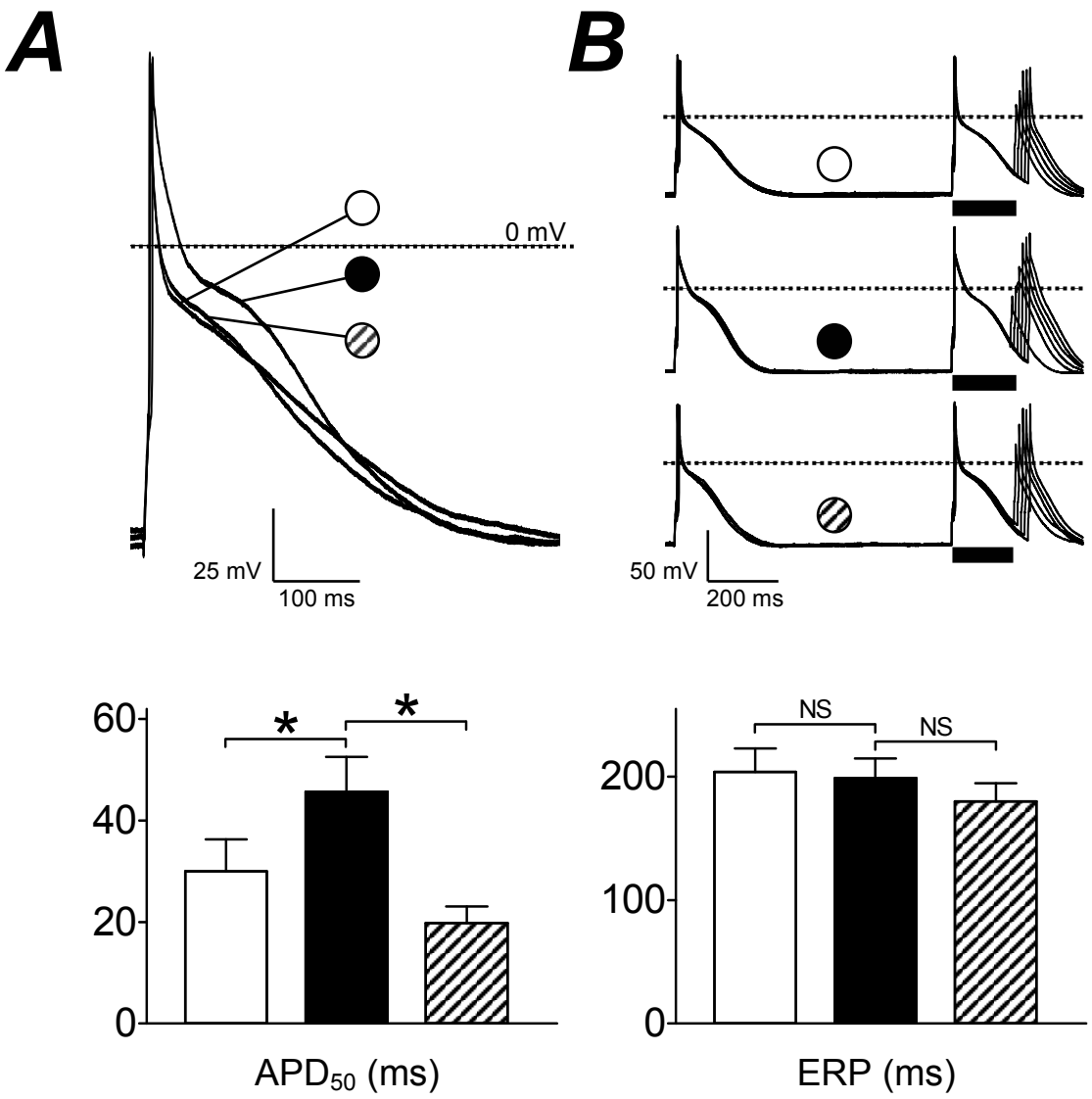


Figure 4.28 Anti-adrenergic effect of ET-1 on action potential early repolarisation, but not refractory period. A, Action potential waveform and duration at 50% repolarisation (APD_{50}) and B, Restitution and effective refractory period (ERP) in atrial cells, in absence of a drug (○), following 0.05 μM ISO (●) and subsequent co-application of 10 nM ET-1 (▨). Upper panels: original, superimposed action potentials, each from the same cell, produced by (A) the 7th of a train of conditioning current pulses (75 beats/min) and (B) 7th and 8th conditioning pulses followed by progressively premature test pulses. Horizontal bars= cell ERP. Lower panels: histogram data; paired means (n=15 cells, 10 patients). Values are mean± s.e.m. Figure taken from Redpath et al, JMCC 2006; 40: 717-724.

not occur under control conditions, were significantly associated with exposure to ISO and following the co-application of 10 nM ET-1 with 0.05μM ISO were significantly abolished (control SD 0% vs ISO SD 100% vs ISO/ET-1 SD 0%, n= 5 cells, 3 patients, $\chi 2$ test, $p< 0.05$ Figures 4.20 & 4.29).

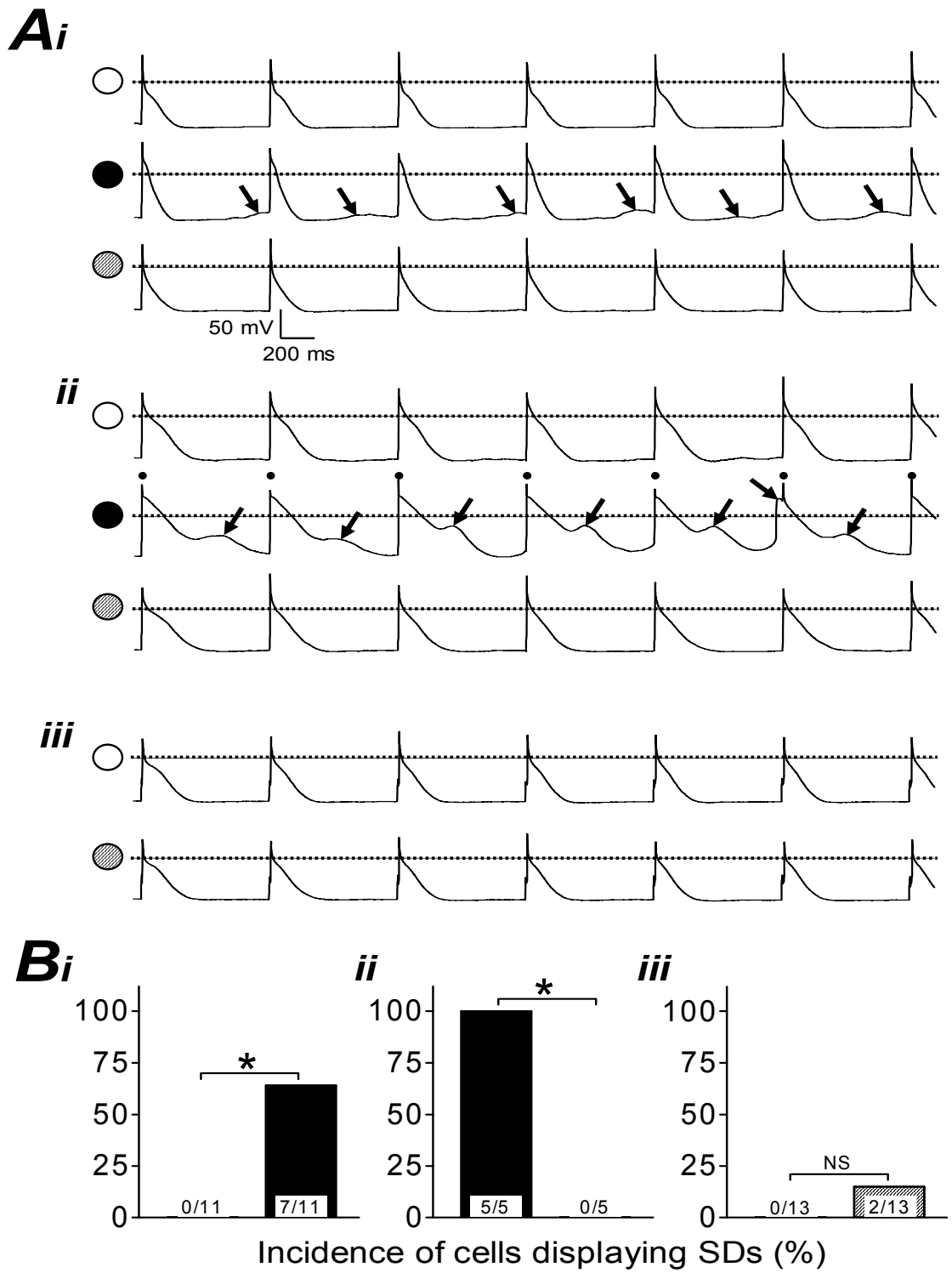


Figure 4.29 Spontaneous depolarisation-induction by isoproterenol, and anti-adrenergic effect of ET-1. A, Original action potentials (APs) evoked by trains of current pulses (75 beats/min) in the absence of a drug (○), with 0.05 μM ISO (●) and subsequent 10 nM ET-1 (▨) in (i) a cell with a normal AP plateau, and (ii) a cell with relatively large plateau. Arrows indicate "spontaneous depolarisations" (SDs): any AP phase 3 transient depolarisation or phase 4 depolarisation >3 mV. Dots indicate driven beats. iii, typical absence of response to ET-1 without ISO. All initial APs are post-rest. B, Histograms showing significant production of SDs by ISO (i), abolition of SDs by ET-1 (ii), and lack of significant (NS) SD-induction by ET-1 alone (iii). Numbers within columns=n cells displaying SDs/total studied. Figure taken from Redpath et al, JMCC 2006; 40: 717-724.

4.6 Discussion

4.6.1 Methodological considerations and action potential morphology

Action potential characteristics as displayed were in accordance with those published previously. There have previously been a number of descriptions of differing human atrial myocyte action potential morphologies[381]. More recently a consensus is emerging of three types of action potential morphologies demonstrated in human atrial RAA specimens has been reached[381;419-426].

Type 1 or typical adult human atrial myocyte action potentials demonstrate a spike and dome morphology and have been previously reported to have a maximal diastolic potential (MDP) of -71 to -86 mV broadly in line with characteristics mathematically modeled from ion current data[421;427].

A second type of action potential morphology was described as having a spike but no dome, reported to be due to the continued presence of I_{TO} but reduced I_{Kr}, related to the enzymatic isolation method[419;420;422-425]. At physiological stimulation rates of 60 bpm there were no significant electrophysiological difference in electrophysiological characteristics between these two action potential morphologies[425]. Mathematical modelling of the human atrial myocyte action potential provides support for this assertion and determines that at physiological heart rate and temperature the action potential morphology is primarily effected by I_{CaL}, I_{TO} and I_{Ksus}[421;427].

A third, infrequent action potential of triangular morphology (absent spike and dome), previously associated with infants with congenital heart disease[420] and described in ventricular myocytes from hearts removed at cardiac transplantation,

was not identified in this study[425]. It was expected that had cells displaying this action potential morphology been encountered they would likely be excluded as at stimulation rates greater then 60 bpm, these myocytes have previously been found to become unresponsive due to the prolonged APD at rest exceeding the PCL[425].

Initial depolarisation, or phase 0 characteristics, of atrial myocyte action potentials have varied among investigators: V_{max} of 149 V/s to 244 V/s, an upstroke amplitude >90 mV and an overshoot of 48 to 53 mV have been reported[79;144;284;419;420;422-425;428-435]. Mean data for phase 0 characteristics in this study are within this published range (See Table 1).

Repolarisation and refractoriness characteristics of human atrial isolated myocytes are particularly susceptible to methodological variation; the APD and ERP physiologically adapt to changes in the cycle length of stimulation and a reduction in environmental temperature accelerates human atrial myocyte repolarisation at identical PCL[72;79;161;419;436]. At physiological stimulation rates of 60-75 bpm the range of repolarisation was found to be APD_{50} of 12 to 62 ms, an APD_{75} of 118 to 138 ms, an APD_{90} between 341 and 447 ms and refractoriness (ERP) of 184-244 ms[79;144;284;419;420;422-425;428-435]. Our data, obtained with a stimulation rate of 75 bpm with the myocytes maintained at physiological temperature, was within this published range (See Table 1).

The two differing morphologies of action potential, although similar, could potentially be explained by heterogeneity in I_{Kr} and I_{TO} as a result of enzymatic isolation or between cells from differing regions of the heart[437], albeit that in the present study all cells were isolated using identical methodology and all tissue samples were obtained from the RAA of consenting patients undergoing corrective cardiac surgery. No direct measurements of I_{Kr} and I_{TO} were made in this study

and further work will be required to resolve this issue. Alternative explanations such as varying disease states and pharmacological therapy will be discussed below.

4.6.2 Patients' characteristics and action potential characteristics

This study was not prospectively powered to determine potential associations between patients' clinical characteristics and human atrial isolated myocyte electrophysiology. The data emanating from this study were pooled with data from colleagues in our laboratory and forms the results section of our recently published manuscript[300]. What has been presented herein represents univariate analysis of the data solely from this project which was performed in order to inform multivariate analysis of our laboratories collective efforts. This, limited, data has been discussed in order to generate hypotheses which were later tested as part of the collective effort[300].

Patient senescence had no significant effect on human atrial action potential characteristics in line with previously published reports from this laboratory[79;144;284]. This is supported by data from experiments examining I_{Na} activation suggesting little if any data supports an alteration in I_{Na} as a function of age bar small shift in the half inactivation potential towards less negative potentials[381;438-440].

Male gender was associated with an increased phase 0 amplitude, however there exists no precedent for this in the literature. Gender has not been identified in previous work on human atrial action potentials, and in pooled data from this laboratory the association of male gender and an increased phase 0 amplitude has not been replicated, suggesting that this may be a type 2 error[79;144;284].

The effects of chronic therapy with β-B on I_{CaL} and action potential repolarisation and refractoriness have recently been described in humans[144;284]. The prolongation of APD_{90} and ERP in the absence of an effect on I_{CaL} was identified on univariate analysis, persisted on multivariate analysis and was found to be independent of heart rate[144]. However, in this smaller patient dataset, although the recorded measurements of repolarisation and refractoriness in this study are consistent with that published by our laboratory, no statistical difference myocyte repolarisation or refractoriness was observed. Thus apparent inconsistency may be explained by the lack of a significant decrease in heart rate related to β-B use (heart rate BBY 58.8± 2.8 v. BBN HR 64.7± 2.8 bpm p= ns, N_p= 13 BBY and N_p= 7 BBN). The lack of a significant heart rate response may be a corollary for underdosing, medication error or non compliance with chronic β-B therapy pre-operatively rather than any absence of effect of β-B on I_{TO}, APD_{90} or ERP[144]. Another explanation is that this inability to reject the null hypothesis represents a type 1 error as pooled data from our laboratory including data from this study, with a significantly larger dataset has maintained the association between β-B therapy and an approximate 20% reduction in APD_{90} and ERP[300].

Pre-operative CCB drug therapy was associated with more rapid early (APD_{60}), mid (APD_{75}) and late (APD_{90}) repolarisation but no effect on ERP in the absence of a direct effect on I_{CaL}. The apparent effect of chronic CCB therapy on mid and late repolarisation, combined with a possible effect on APD_{50} which approached statistical significance (p= 0.051) prompted the measurement of APD_{60}. Both Diltiazem and Verapamil have previously been associated with acutely reducing the AP plateau as recorded by APD_{50}[430;441]. Chronic pre-operative therapy with CCB has also been associated with reduced I_{CaL} and accelerated repolarisation[282]. However, in contrast, a larger study in this laboratory confirmed a lack of effect of chronic CCB therapy on I_{CaL}[144]. In addition no significant effect on APD or ERP

was observed[144]. Patients in both studies form this laboratory were predominantly receiving dihydropyridine (Amlodipine or Nifedipine) CCBs pre-operatively at similar dosages. After merging the datasets the apparent effect of CCB therapy on action potential repolarisation ceased to be statistically significant on univariate analysis.

ACE-I therapy was associated with a small increase in action potential upstroke velocity (V_{max}) during phase 0 depolarisation but no other significant effects on action potential characteristics. Data from multicellular recordings in animal models suggestive of a role for AII antagonism reducing cellular and structural remodelling such as atrial fibrosis may suggest a link, however there is no data from single cellular recordings to suggest a direct effect of ACE-I therapy on I_{Na}[118;442-446]. However I_{Na} was not measured in this study and thus would require further work to determine the presence of any direct effect on I_{Na} and the reproducibility of any effect on V_{max} in human atrial myocytes.

Initial reports describing human atrial action potentials correlated diseased, dilated atria with a depolarized MDP, however no specific associations between atrial AP characteristics and disease processes have been identified[115;430;432;433;447;448]. Data on atrial dimensions were not available for this study, however an analysis of the effect of LVSD was made and a small but significant hyperpolarisation of the MDP was observed. This had no apparent effect on phase 0 depolarisation characteristics and this has not previously been reported. No effect was identified for LVSD nor prior MI on any other action potential characteristic in this study.

The possible effects of LVSD on human atrial myocyte repolarisation and refractoriness are controversial with previous reports describing no change in early[449;450] or late[451] repolarisation, accelerated early and late repolarisation[451] and

prolongation of APD_{90}[449;450]. The present study failed to identify any significant association with altered repolarisation or ERP but in order to resolve the controversy, pooled data from our lab, subject to multivariate analysis and incorporating data relating to patients with objective assessments of LVSD identified that LVSD is associated with abbreviated APD_{90} and ERP by approximately 10-20%.

Similarly controversial to the possible impact of LVSD on atrial I_{CaL} and atrial action potential characteristics is the possible interplay of effects of ISO and ET-1 on I_{CaL} and action potentials in the presence and absence of LVSD. The next Results chapter will build on the data presented in this chapter on action potentials under control conditions by investigating the effects of acute superfusion of ISO and ET-1, alone and in combination, on human atrial action potentials.

4.6.3 Effect(s) of ISO on action potentials

The first experimental recordings of human atrial action potentials almost 50 years ago were able to demonstrate that adrenergic stimulation with epinephrine or isoproterenol increased the action potential plateau and caused afterdepolarisations without alteration of phase 0 characteristics[419;428;429;452] as observed in this study. The presence or absence of β-B therapy was not associated with alteration in action potential characteristics, also consistent with our findings (See Chapter 3 Results section). Due to the small sample of recordings further sub-analysis of the association with prior β-B therapy modulating either the ability of ISO to elevate the action potential plateau or to induce SDs is not possible. The lack of effect on later repolarisation and cellular ERP, in combination with the known effect of ISO to augment I_{TO} and I_{Kur} suggests that in addition to increasing APD_{50} there must also be ‘compensation’ of late

repolarisation in order to fully repolarise 'on time' and to preserve ERP[164;165;424;453]. It also suggests that SDs are related to elevated I_{CaL} rather than some hithertofore unappreciated effect on K repolarising currents as the SDs occur in cells in which there has not been lengthening of ERP. This suggests that, at least insofar as human atrial isolated myocytes in vitro can be described to undergo arrhythmogenesis, triggered activity, traditionally associated with arrhythmia initiation, is a potential arrhythmogenic effect of adrenergic stimulation.

4.6.4 Effect(s) of ET-1 on action potentials

ET-1 has been noted to enhance I_{Na} in animal studies and a small number of human atrial myocytes associated with a hyperpolarized resting membrane potential and an increased amplitude and upstroke velocity of phase 0 of the human atrial action potential[176;200;261]. In contrast a small but significant reduction in V_{max} in the absence of an effect on MDP was detected in this the largest study to date of the direct effect of ET-1 on human atrial myocyte action potentials at physiological temperature with preserved cellular integrity. As no recordings of I_{Na} were made in this study we can only speculate as to a potential reduction in I_{Na} associated with acute superfusion with 10 nM ET-1. Methodological differences such as a 10 mV less depolarized holding potential in this study as compared to prior studies of I_{Na} may, in part, explain the difference in results. Further direct measurements of the effect of ET-1 on I_{Na} would be required to resolve this question.

Initial reports described stimulatory[196;248;258;259], inhibitory[195;196;248;260-263] or no effect[203;247;249;264] of ET-1 on I_{CaL}, with an EC_{50} between 1 and 10 nM, mediated via $ET\text{-}_A$ receptors in animal models[195;196;203;248;249;258-264]. In human atrial myocytes, ET-1 alone was found to increase[199], decrease[199;200] or have no effect[199;200] on I_{CaL}

under control conditions. The action potential data in this study confirms the earlier finding (See Results Chapter 3) that under conditions of physiological temperature with a ruptured patch preserving human atrial myocyte integrity 10 nM ET-1 alone has no effect on I_{CaL}. In human right atrium ET-1 was demonstrated to have little or no effect on cAMP under basal conditions, but significantly inhibited adenylate cyclase due to ET-$_A$ receptor/ $G_{\alpha i}$/ IP_3 formation independent of I_{CaL} and PKC following adrenergic stimulation[187;200]. Indeed the anti-adrenergic effect of ET-1 is abolished during simultaneous intracellular application of 8-Br-cAMP[200].

4.6.5 Effect(s) of ISO and ET-1 in combination on action potentials

In accordance with the data presented herein, adrenergically prestimulated I_{CaL} has been consistently found to be inhibited by 10 nM ET-1 via ET-$_A$ receptors acting on $G_{\alpha i}$ (pertussis sensitive) GPCRs[245;247;262] reducing cAMP in human atrial myocytes in the absence of any direct effect on the L-type calcium channel itself[198-200].

In addition to an anti-adrenergic effect of on I_{CaL}, ET-1 has been reported to activate NHE[179;241;265;393] and NCX[241;266;268;393] via PKC and inhibit I_{Cl} via reducing intracellular cAMP[205;273]. This would be expected to indirectly affect I_{CaL} because of increasing intracellular calcium levels which may explain the positive inotropic effect of ET-1[174;197;265;271;382-384;454;455] in the absence of a simultaneous increase in I_{CaL}[179;197;382-384]. However an increased intracellular calcium transient[192;197;264;267;392;456;457] despite the sensitisation of myofibrils to calcium[265;384] has previously been associated with the generation of SDs[319;391;392;457;458]. The data presented do not support this, however again in the absence of direct measurements of the ion currents this possibility cannot be discounted.

Endothelin has also been reported to have direct effects on in human atrial myocyte repolarising potassium channels[371] which also contribute to determining the amplitude and duration of the action potential[459]. ET-1 induced marked inhibition of I_{K1} via PKC in human atrial myocytes at physiological temperature using whole cell patch clamp[371]. A reduction in an inward rectifying current necessary for maintaining the resting membrane potential of atrial myocytes may induce early or delayed afterdepolarisations and abnormal automaticity[270;272]. In animal studies ET-1 modulation of K^+ outward currents is conflicting[62;269-272]. In various models ET-1 was observed to increase the delayed rectifier I_K[264;456], and I_{Kss}[271;454] however also to inhibit I_K[62;271], I_{Kss}[271;454], I_{TO}[417], I_{KACh}[269;460] and I_{KATP} via $ET\text{-}_A$ receptors. Data on the resulting effects on action potential repolarisation are also mixed with no change[454], an increase[383] and a decrease[61] in APD reported. The present study does not support, but cannot exclude, a direct effect of ET-1 on K^+ currents in human atrial myocytes. The lack of change in action potential repolarisation with superfusion of ET-1 alone, the lack of spontaneous depolarisations following exposure of myocytes to ET-1 and the 'symmetrical' anti-adrenergic effect of ET-1 co-application following exposure to ISO all would suggest an ET-1 effect predominantly limited to I_{CaL}.

In summary, in contrast to ISO, 10 nM ET-1 did not appear to be associated with any arrhythmogenic effect on human atrial isolated myocytes in vitro. Indeed ET-1 could be viewed as being anti-arrhythmic as the pro-arrhythmic effect(s) of ISO were abolished by the addition of 10 nM to the ISO containing superfusate. It is appealing, but premature, to ascribe all the anti-arrhythmic effects of ET-1 as a direct translation of the anti-adrenergic effect on I_{CaL} through to early repolarisation and SDs.

5 GENERAL DISCUSSION

5.1 Main Conclusions

The data presented herein confirm that under these experimental conditions ET-1 modulates the effects of ISO on I_{CaL} and action potentials via ET_{-A} receptors in human atrial isolated myocytes. There was no apparent effect of ET-1 alone on either I_{CaL} or action potential repolarisation or refractoriness and the anti-adrenergic effects of ET-1 were not modified by chronic pre-operative β-B therapy. Whether the anti-adrenergic effects of ET-1 are anti-arrhythmic in humans is yet to be determined.

The anti-adrenergic effect of ET-1 on I_{CaL} was observed at concentrations of ET-1 which have been reported in the venous blood of patients suffering from severe CHF. This ET-1 effect was prevented by the ET_{-A} receptor anatagonist, FRA, and was partially reversible both by washout and subsequent superfusion with FRA.

ET-1 alone was associated with a reduction in V_{max} but not repolarisation or refractoriness. ET-1 did however reverse the adrenergically induced increase in APD_{50} and spontaneous depolarisations.

The possible physiological mechanisms of these effects, such as secondary messenger systems and interactions with other atrial ion channels, and their potential implications for arrhythmogenesis in human atria will be discussed.

5.2 Possible physiological mechanisms of the effects of ET-1 in human atrial myocytes

ET-1, binding to ET-$_A$ receptors expressed on cardiac myocytes has a number of biological effects mediated via stimulation of Gα_i[396-398]: ET-1 was found to directly inhibit or reduce cAMP[187;246;261;399] and increase InsP3[187;384;391;392;455;458] with direct experimental evidence for anti-muscarinic[269] and anti-adrenergic effects[187;246;249;261;262] in a number of animal models.

In human atrial myocytes evidence exists for a basal constitutive cAMP/ I_{CaL} activity[281;287;288;395]. Upon binding to β-AR, ISO rapidly induces a threefold rise in cAMP activity which, via PKA-induced phosphorylation of I_{CaL} results in a sustained elevation of long lasting inward calcium current[90;238;239;302-307]. ET-1, with an EC_{50} of 1-10 nM, binds to the ET-$_A$ receptor on human atrial myocytes rapidly stimulating Gα_q activating PLC which hydrolyses phosphatidylinositol present in the plasmalemma. The resultant 1,4,5-inositol triphosphate ($InsP_3$) enters the cytosol and diacyl-glycerol (DAG) remains in the plane of the membrane[327;457;458;461;462]. In human right atrial specimens, ET-1 was not found to have any effect on cAMP levels under basal conditions but could reversibly inhibit an adrenergically-activated adenylate cyclase/ cAMP cascade[187;200] the effects of which were not limited to I_{CaL}[199;200]. In addition, within seconds, PKC (δ and ε)[313] is activated[462;463] and translocates close to the membrane to activate small Ras G proteins and extracellular signal related kinase (ERK) within 3 minutes[464]. $InsP_3$ also stimulates SR calcium release resulting in a rapid increase in $[Ca^{2+}]_i$, independent of external calcium, followed by a sustained increase in $[Ca^{2+}]_i$ facilitated by increased SR calcium uptake[457;458;465-467].

ET-1 binding to ET-$_A$ also activates phospholipases A and D, altering arachidonic acid metabolism and providing sufficient supply of DAG for sustained PKC activation[468;469]. PKC then selectively phosphorylates the α_{1c} subunit of I_{CaL} resulting in decreased calcium entry[90]. Recently, the possibility that ET-1 induced upregulation of PKC may play a role in the augmentation of constitutive I_{KACh}, increased in AF and a cause of APD abbreviation, and I_{KATP} has been raised[470-473].

Alternative second messenger systems may be activated by ET-1 directly. Evidence is accumulating that, in addition to an inhibitory action on cAMP via $G_{i/o}$[396-398], the anti-adrenergic effect of ET-1 is via stimulation of a phosphatase which opposes the PKA-phosphorylation of calcium handling regulatory proteins[242;245] independent of PKC activation[242;248]. Human atrial I_{CaL} has been shown be modulated via the cGMP cascade, albeit that the overall effect varies with the relative balance of the three cGMP effector proteins[281;287;288;380;395]. Modest activation of cGMP increases cAMP via inhibiting PDE3 resulting in a positive inotropic effect and an increase in I_{CaL}, however strong activation of cGMP produces both an activation of PDE2, opposing PDE3, and activation of PKG resulting in an overall inhibitory effect on I_{CaL}[287].

A further level of complexity in understanding the effects of ET-1 is due, in part, to the crosstalk present between the angiotensin, adrenergic and endothelin receptors' secondary messenger systems[243;248;474;475]. Endothelin is synthesised, stored and released in human atrium and crosstalk between AII and ET-1 as an autocrine phenomenon has been described[474].

In summary it appears that the possibilities for an ET-1 mediated anti-adrenergic effect on I_{CaL} and subsequently action potentials are as follows: ET-1 stimulates calcium release from the SR producing calcium mediated inactivation of

I_{CaL}[195;197;258], ET-1 stimulates IP_3 formation and in so doing inhibits cAMP formation by adenylate cyclase reducing the ability of cAMP dependent kinases to activate I_{CaL}[187;188;200;262;273;399], or that ET-1 stimulates cyclic nucleotide phosphodiesterase via cGMP hydrolyzing cAMP antagonizing the adrenergic effect of β-AR stimulation[187;199;200;242] on I_{CaL} but not NCX[402;403]. The apparent contradiction in an inhibitory effect on I_{CaL} but a positive inotropic effect could be explained by the relative efficacy of the differing second messenger systems effected by ET-1 binding to $ET\text{-}_A$ receptors: sensitizing the SR and increasing cytosolic calcium during diastole will oppose calcium entry via I_{CaL}[195;197;258], PLC activation and IP_3/ DAG stimulation produces a positive inotropic effect but the associated inhibition of cAMP will reduce I_{CaL} amplitude[187;188;200;262;273;399] and activation of NHE[265] and NCX[266-268] via PKC will result in a positive inotropic effect with little direct effect on I_{CaL}.

5.3 The potential effects of ET-1 on the fundamental mechanisms of arrhythmogenesis in human atria

The three fundamental mechanisms underpinning arrhythmogenesis are triggered activity, reentry and abnormal automaticity. The occurrence of these fundamental mechanisms is promoted by several pathophysiological remodelling processes (Figure 1.1). The potential for ET-1, alone or in combination with ISO, to affect human atrial arrhythmogenesis could be modified by the duration of exposure, local concentration and tissue exposure within the heart, pathological disease states, pharmacological therapy and the presence of physiological stress. Evidence suggesting a possible role of ET-1 in any of these three fundamental processes, and impact on AF or CHF induced remodelling will be discussed.

5.3.1 Triggered Activity

ET-1 was, at first, thought likely to predispose to EADs and DADs by reducing I_K and causing increased intracellular calcium loading[50] via augmenting I_{CaL}. However the data suggesting that ET-1 decreases I_K[62;271] are controversial[264;456] and in the present study and one other ET-1 did not affect APD[322;454]. ET-1 did augment the calcium transient via increased calcium mobilisation from SR and perinuclear stores[192;197;264;267;392;456;457] activating NHE[179;241;265;393] and NCX[241;266;268;393]. This may potentially be pro-arrhythmic, with ET-1 increasing the magnitude of calcium sparks sufficiently to induce diastolic calcium waves correlating with spontaneous depolarisations[319;391;392;458]. However, in an open chest dog model ET-1 had an anti-adrenergic protective effect on ISO induced atrial fibrillation and ventricular arrhythmia[476]. Although the data presented here relate to single myocyte recordings, ET-1 abolished both the early and late spontaneous depolarisations initiated by ISO during action potential recordings[322], directly analagous to the anti-adrenergic phenomenon described in the open chest dog model[476]. Further evidence of a potential anti-arrhythmic effect of ET-1 comes from clinical trials in human patients of long term $ET\text{-}_A$ receptor blockade. Despite promising results in animal testing[274;275], results of clinical trials in human patients with CHF have been disappointing[276] with two larger studies being terminated prematurely due partly to an unexpected increase in atrial[277] and ventricular[278] arrhythmia.

The acute effects of ISO on I_{CaL} and I_K translate into altered action potential repolarisation and refractoriness. Data from early experiments in line with the data presented here confirm that acute superfusion with ISO increased the action potential plateau and caused afterdepolarisations without alteration of phase 0 characteristics[419;428;429;452]. The lack of effect on later repolarisation and cellular

ERP, in combination with the known effect of ISO to augment I_{TO} and I_{Kur} suggests that in addition to increasing APD_{50} there must also be 'compensation' of late repolarisation in order to fully repolarise 'on time' and to preserve ERP[164;165;424;453]. It also suggests that SDs are related to elevated I_{CaL} rather than some hithertofore unappreciated effect on K repolarising currents as the SDs occur in cells in which there has not been lengthening of ERP. This is also suggested by the lack of effect of ET-1 alone on I_{CaL} and the absence of a significant effect of ET-1 alone to cause SDs. This suggests that, at least insofar as human atrial isolated myocytes in vitro can be described to undergo arrhythmogenesis, triggered activity, traditionally associated with arrhythmia initiation, is a potential arrhythmogenic effect of acute adrenergic stimulation and that this arrhythmogenic effect is opposed by ET-1.

Unopposed β-AR agonism, such as that experienced during decompensated CHF, would be expected to increase both I_{CaL} and I_{Kur} / I_{Ks} and these opposing effects on APD may protect against excessive APD prolongation in human atria[69;165]. Any increase in cAMP or PKA activity will result in increased phosphorylation of calcium handling proteins and augmentation of CHF induced remodelling[69]. Elevated PKA hyperphosphorylates RyR2 disassociating calstabin and CaMKII[477;478] in human ventricular preparations resulting in depleted SR calcium stores because of diastolic calcium leak from the SR[477-479] activating inward NCX and potentially culminating in triggered arrhythmia[210]. The potential arrhythmogenic effect of leaky RyR2 in CHF is only beginning to be investigated[477;480-483], however. Fortunately, although Ca ATP-ase (SERCA2a) and phospholamban (PLB) levels remain unaltered, PLB phosphorylation is decreased because of subsequent increased phosphatase activity in CHF[484-488]. This results in decreased calcium uptake, SERCA2a function and CaATP-ase activity[479;485;487;489-494] reducing subsequent calcium transient and rate of decay,

culminating in a reduced force frequency relationship[491;495] and a lower probability of triggered activity.

Despite this adaptation, in animal models adrenergic stimulation and altered phosphorylation status of PKA and CaMKII in combination appear to result in sufficiently leaky SR calcium release channels to produce diastolic calcium waves which can result in triggered arrhythmia[43;496;497] with evidence of altered CaMKII activity in human CHF[479], despite reduced myocyte calcium stores[210;496] because of hyperphosphorylated SR calcium release channels, the electrogenicity of calcium extrusion by NCX and the increased membrane resistance due to decreased I_{K1} resulting in greater depolarisation for any given inward current[69].

AF-induced electrical remodelling is accompanied over a longer time course by cellular remodelling[98]. AF has been associated with human atrial myocyte apoptosis[96] and the surviving atrial myocytes demonstrate significant ultra-structural abnormalities[97;498;499]. Atrial myocyte degeneration with sarcoplasmic reticulum fragmentation, marked collagen formation, widening of the intercalated discs and replacement of myofibrils results in patchy fibrosis, atrial dilatation and myopathy[97;498;499]. More frequent calcium sparks and calcium waves were observed in atrial myocytes isolated from patients in AF when compared to those from patients in sinus rhythm despite apparently similar levels of SR calcium loading and greater NCX activity suggesting a direct role for the calcium release channel[16]. Elevated levels of PKA induced hyperphosphorylation of RyRs resulting in dissociation of calstabin2 (FK506) and thus increasing the P_o due to calcium hypersensitivity could also contribute to increased leak of calcium from the SR in human atria initiating AF via triggered activity or supporting arrhythmia maintenance in patients already suffering AF[16;477;500].

5.3.2 Intra-atrial Reentry

Intra-atrial reentry is promoted by any factor which reduces conduction velocity and refractoriness or increases path length or conduction time. Thus decreased I_{Na}/ V_{max}, decreased I_{CaL} or increased I_K, decreased APD and ERP, atrial dilation and stretch all promote reentry. We did not measure I_{Na} or I_K directly and therefore can only speculate on the direct effect of ET-1 on these ion currents. The small reduction in V_{max} following acute superfusion with 10 nM ET-1 in this study suggests a possible decrease in I_{Na}, however this is contrary to previous reports in human right atrial myocytes[176;200;261] and should interpreted with caution. The lack of effect of ET-1 on APD suggests that in the present model ET-1 does not acutely affect I_K. However, one study of human atrial myocytes reported that ET-1 induced a marked inhibition of I_{K1}[371]. In view of the absence of an ET-1 effect on APD and ERP in this study, at the very least it can be said that using this model of arrhythmogenesis there is little or no evidence for ET-1 alone to promote intra-atrial reentry via a direct effect on ion channels or refractoriness in human atrial isolated myocytes.

Heart Failure, whether acute[501] or chronic[502] results in left atrial stretch and dilatation which has been associated with reductions in I_{CaL}, APD shortening and plateau depression[115]. Myocytes isolated from dilated human atria are larger with smaller I_{CaL} and demonstrate abbreviated APD, perhaps due to elevated levels of ANP, altered redox state or kinase/ phosphatase activity[84;115;199;299;379;503]. Whether this reduced I_{CaL} translates into a reduction in atrial ERP is unclear[115;117] however the relationship between atrial dilatation, altered atrial electrophysiology and prognosis appears robust[115;504;505]. Certainly atrial dilatation and calcium overload as a result of tachycardia will result in oxidative stress, as indicated by glutathione

depletion and increased NADPH activity which have been linked with impaired contractility, reduced I_{CaL} and I_{Kur} AF in man[97;375;377;379;506-508].

Altered phosphorylation as a result of stretch-induced oxidative stress can also modulate the response of I_{CaL} and cellular calcium processing to adrenergic stimulation[84;379;509;510] perhaps directly via the RyR and L-type calcium channels[374;376;379;395;511]. This mechano-electric feedback[512;513], demonstrated in humans[514] and animal models[515-517] via vagal efferent stimulation, results in electrical instability by accelerated repolarisation and ERP shortening[518] further predisposing to arrhythmia[519]. Acute atrial stretch may have variable effects throughout the atria thereby generating non uniformity of conduction and this combined with increased atrial size is more likely to permit accommodation of the multiple daughter wavelets seen in fibrillating atria[520] promoting reentry or enhance the automaticity of arrhythmogenic foci[448].

CHF, in addition to direct cardiac electrophysiological remodelling effects[69;114;115;117;119;158;449], is associated with increased levels of circulating catecholamines and adrenergic tone which in turn can have profound effects on human cardiac performance[122;151;255;331;332;521-523]. In human atrial myocytes acute exposure to ISO has a concentration dependent effect via β-AR stimulation increasing I_{CaL}, I_K and I_{Kur} via PKA/ cAMP, and α-AR stimulation by Phenylephrine decreases I_{Kur} and I_{to} via PKC[165]. This indicates that the repolarisation prolonging effect of ISO by increasing I_{CaL} and inhibition of I_{to} outweighs opposition by stimulatory effects on I_{Kur}[164;165;321;424].

In the present study, no measurements of K^+ currents were made but acute superfusion with ISO augmented I_{CaL} in a concentration dependent manner without altering the I:V relationship, activation or inactivation kinetics in human atrial

isolated myocytes. This adrenergic increase in I_{CaL} has previously been attributed to an increased mean channel opening time and an increased P_o[90;238;239;302-307].

The acute effects of ISO on I_{CaL} and I_K translate into altered action potential repolarisation and refractoriness. Data from early experiments in line with the data presented here confirm that acute superfusion with ISO increased the action potential plateau and caused afterdepolarisations without alteration of phase 0 characteristics[419;428;429;452]. The lack of effect on later repolarisation and cellular ERP, in combination with the known effect of ISO to augment I_{TO} and I_{Kur} suggests that in addition to increasing APD_{50} there must also be 'compensation' of late repolarisation in order to fully repolarise 'on time' and to preserve ERP[164;165;424;453].

5.3.3 Abnormal Automaticity

Abnormal automaticity in the atrium occurs when atrial or pulmonary vein myocytes, which are not normally pacemaker cells, begin to spontaneously fire at a faster rate than the SAN as a result of accelerated phase 4 depolarisation (See Section 1.2.3).Although fractionation or fibrillatory conduction has been observed in patients, no direct evidence for a role of abnormal automaticity has been identified to date in patients[67-69]. Currently there is no data to suggest that ET-1 has an effect on I_f or phase 4 of the human atrial action potential.

5.4 Limitations of this work

Despite the obvious scientific benefits, there are inherent practical and theoretical limitations in studying human tissues. The availability of tissue is limited, by virtue that cardiac tissue is only available at the time of cardiopulmonary bypass during cardiac surgery, a small sample of tissue is obtained and thus fewer cells are

available for experiments. In addition the confounding effects of competing comorbidities and pharmacological therapies, effects of age and gender and hithertofore unknown other factors can potentially limit the applicability of some experimental results.

The enzymatic dissociation method is recognised to result in cells which are depolarised at rest after experimentation. Furthermore, despite strenuous attempts to maintain and protect the myocytes, prolonged recording protocols are limited, despite the success of perforated patch recordings.

5.5 Future Directions

AF remains the most common disorder of cardiac rhythm[1;524-526], affecting more than half a million people in the UK alone[527] and estimated to cost the UK NHS more than £1 Billion in 2000[527]. The prevalence of AF in the UK has risen[528] and will continue to do so as the population ages[1;529] and improvements in the management of associated conditions such as CHF are implemented[530]

AF results in several deleterious, yet reversible, effects on cardiac physiology: loss of Atrio-Ventricular synchrony and rapid irregular ventricular systole negatively effect cardiac output[2;3;531-535] increasing the risk of atrial[536] and ventricular tachycardiamyopathy[537] and ultimately CHF[538-540]. Even in the absence of coronary atheroma, AF increases myocardial oxygen demand whilst simultaneously decreasing coronary perfusion resulting in myocardial ischaemia[541;542] and hence potentially precipitating further arrhythmia[543].

These experiments have prompted me to consider investigating the links between the intracellular calcium handling effects of ET-1 and alterations of I_{CaL} and action potential characteristics at the plasmalemma. Experiments performed with

confocal microscopic measurements would provide further insight into the effects of neurohormonal activation on human atrial myocytes but also the multifaceted process of atrial excitation contraction coupling. This work would enhance our knowledge of the generation of EADs and DADs. Expansion to multicellular preparations would allow further assessment of the ability of adrenergically induced afterdepolarisations to be propagated linking to arrhythmogenesis. The interaction between other neurohormones, such as ANP and AII, and catecholamines and ET-1 may prove another fruitful piece of experimental work. Do these autocrine/ paracrine substances interact at the receptor level, crosstalk, or within the cell at the level of secondary messengers or indeed via complementary or opposing effects on calcium handling, or indeed all of the above. If such interaction exists is this pro or anti arrhythmic?

Expanding the area of interest into the K^+ currents involved in repolarisation may also permit further insight into atrial myocyte electrophysiology. Although investigation of I_{Kr} and I_{Ks} in human atrial myocytes is difficult due to the chunk method of isolation[544], an investigation into the impact of ET-1 on these important repolarising currents is much needed.

Investigation of a potential role for tyrosine phosphorylation and PKC in the modulation of I_{CaL} in human atrial myocytes, whether membrane bound or cytosolic, warrants further examination. Interest in reports of a role of Ca2+/ calmodulin dependent protein kinase II (CAMKII) activity in modulating I_{CaL} and RyR function have been aroused by the data relating to the role of protein phosphatases in AF and CHF induced structural remodelling[84;483;496;497;545-547].

By the very nature of the experimental method, studies of an acute exposure of myocytes to single, biologically plausible, contender molecules, such as ISO and

ET-1 in this study, have commonly been performed. The possibility of performing integrated physiology experiments in animal models examining the effect(s) of chronically elevated ET-1 levels on atrial and ventricular electrophysiology should be explored. A comparison with those electrophysiological effects occurring in cardiac tissue following an induced heart failure syndrome would assist in the understanding of the links between CHF, AF, ion currents and calcium handling.

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Appendices
(i) Patient consent form

Right atrial appendage

Glasgow Royal Infirmary University N.H.S. Trust

PATIENT INFORMATION AND CONSENT FORM

Title of Project: Electrophysiological and molecular properties of isolated human cardiac myocytes.

Summary:
You are shortly to undergo a heart operation. The reasons for this, and the type of operation to be performed, will have been discussed with you by your cardiologist and by the heart surgeon.

We are undertaking research into why some patients have abnormal heart rhythms and how these cardiac arrhythmias affect the heart cells. In order to understand the biological properties of heart cells we would like to obtain a small sample of your heart muscle.

During the operation, the surgeon routinely makes a small cut in the heart to allow the operation to proceed. Many surgeons perform this by removing a small piece of heart tissue (about the size of a pea), which would normally be discarded. Some surgeons do not routinely remove this piece of tissue, but are willing to do so, since they know that such a procedure would not do you any harm. We are asking for your consent to use this small piece of the heart to obtain heart cells for our study. Part of the tissue will be studied directly for electrical activity and then discarded, and the remainder will be deep-frozen at the hospital for future analysis of molecular activity, after which it will be destroyed. Participation in our study will not change the operation or result in additional risk or discomfort to you.

In addition, we would ask your permission to obtain details of your condition from your medical records. These details will be kept confidential and your identity will be concealed by the use of a coding system. Such information will be retained for comparison with the results of the tissue analysis, and will not be disclosed to anyone outside the hospital.

This research project will not be of direct benefit to you, but the results may help other patients in the future. If you are unwilling to take part, you are entirely at liberty to refuse permission. This would not affect your medical treatment in any way.

Consent:

I, (Name) …….

of (Address) ……

agree to take part in the Research Project described above.

Dr …….. has explained to me what is involved, how it might affect me and the purpose of the Research Project.

Signed …… Date ……..

Witness ….…… Date ……..

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