THE EFFECT OF ACTIVATED PROTEIN C ON CARDIAC CELLS

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Abstract

Activated Protein C (APC) is an endogenous anticoagulant that regulates thrombin generation. Its physiological importance is reflected by the thrombotic phenotype of PC deficient individuals and those with APC resistance due to mutation at the Factor V cleavage site. At a molecular level, thrombin binding to the receptor thrombomodulin (TM) converts endothelial protein C receptor (EPCR) bound PC into APC. APC has a negative feedback role in curbing thrombin generation by inactivating coagulation factors V and VIII.

Clinically, acquired PC deficiency occurs in conditions such as severe sepsis as a result of mechanisms involving consumption, increased degradation by proteolytic enzymes and reduced hepatic synthesis. The initial success of APC in sepsis (PROWESS Trial) over other anticoagulants is thought to relate to anti-inflammatory and cyto-protective properties The interaction of EPCR–APC with protease activated receptor-1(PAR-1), the prototypical thrombin receptor appears to be relevant *in vivo*. Intravenous injection of APC into mice leads to PAR-1-dependent gene induction and interestingly gene expression profiling of cytokine-stimulated endothelial cells demonstrates marked differences between APC and thrombin signaling.

Cardiovascular dysfunction occurs frequently in patients with sepsis, increasing the mortality rate significantly from 20% to 70-90%. Key observations from the PROWESS Trial highlighted a possible link between APC and cardiovascular recovery. For those with cardiovascular dysfunction at baseline, APC treatment led to a significantly greater likelihood of organ resolution. Furthermore, in the most critically ill patients with multiple organ dysfunction, time to resolution of cardiac

dysfunction was significantly shorter for APC treated patients with improvements linked to overall mortality reduction.

The precise cause of cardiac dysfunction in sepsis remains unclear. Endothelial activation has been implicated as has decreased contractility and impaired myocardial compliance. Postulated mechanisms include cardiac myofibrillar dysfunction with altered calcium sensitivity and the influence of myocardial depressant factors such as pro-inflammatory cytokines and nitric oxide. The potential effects of APC on the cardiovascular system could be multifactorial.

This thesis sets out to examine if the anti-inflammatory and cytoprotective properties of APC may be involved.

In the first experimental chapter, the effect of APC in free or microparticulate form on human coronary artery endothelial cells was examined. An anti-inflammatory and cytoprotective profile is induced although evidence of reversing the pro-inflammatory effect of tumour necrosis factor alpha (TNFα) was inconclusive.

In the second experimental chapter the effect of APC on cardiomyocyte contraction was studied using rat ventricular cardiomyocytes and this showed the potential role of EPCR and PAR-1.

In view of the high efficiency of PAR-1 activation by thrombin, the third experimental chapter looked at whether any APC effect would continue to be relevant in the presence of thrombin. The APC response was observed at clinically relevant concentrations, without significant modification of calcium handling and despite the presence of thrombin.

This led to the final experimental chapter where PAR-1 mediated effects appear to be regulated by downstream protein kinase C alpha (PKCα) providing mechanistic insight in to the findings

This thesis concludes that APC has functional relevance with respect to cardiac cells both at the endothelial and contractile level. The results offer a framework to delineate distinct pathways for improved therapeutic manipulation in the treatment of myocardial sepsis and depression.

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DECLARATION

The work presented in this Thesis is my own.

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FIGURES

TABLES

ABBREVIATIONS

A1	Apoptotic Bcl-2 homologue
ACE	Angiotensin converting enzyme
ADAMS17	ADAM metallopeptidase domain 17
APACHE II	Acute Physiology and Chronic Health Evaluation II
APC	Activated protein C
aPTT	Activated partial thromboplastin time
Bcl-2	B-cell lymphoma 2
BEC	Brain Endothelial Cell
CAD	Coronary artery disease
CASP	Caspase
CRP	C-reactive protein
cT	Cardiac Troponin
DIC	Disseminated intravascular coagulation
dS	Amplitude of shortening
dS/dT	Maximum velocity of shortening
EAhy926	Human endothelial cell line
ENHANCE	Extended Evaluation of Recombinant Human APC
EPCR	Endothelial protein C receptor
ERK	Extracellular signal-regulated kinase
FACS	Fluorescence-activated cell sorting
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GPCR	G protein coupled receptors
HCAECs	Human Coronary Artery Endothelial Cells
HPAECs	Human Pulmonary Artery Endothelial Cells
HUVECs	Human Umbilical Vein Endothelial Cells

IAP-1	Inhibitor of apoptosis protein 1
ICAM	Intercellular adhesion molecule 1
ICU	Intensive Care Unit
IIa	Thrombin
IL	Interleukins
LCC	L-type calcium channel
LD	lethal dose
LNCaP	Adenocarcinoma of prostate, cell line
LPS	Lipopolysaccharides
LVEF	Left ventricular ejection fraction
MAP	Mean arterial pressure
MAPK	Mitogen-activated protein kinase
MCA	Middle cerebral artery
Mcl1	Induced myeloid leukaemia cell differentiation protein1
MHC	Major histocompatibility complex
MMP	Matrix metalloproteinase
MOD	Multiple organ dysfunction
MP	microparticle
MPO	Myeloperoxidase
NCX	The sodium-calcium exchanger
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NO	Nitric oxide
NOS	Nitric oxide synthase
PAI-1	Plasminogen activator inhibitor-1
PARs	Protease activated receptors
PBL	Phospholamban
PC	Protein C

PCAM	Platelet/endothelial adhesion molecule
PCNA	Proliferating cell nuclear antigen
PDGF	Platelet-derived growth factor
PE	Phosphatidylethanolamine
PFP	Platelet free plasma
PI3K	Phosphoinositide-3-kinase
PKA	Protein kinase A
PKCα	Protein Kinase C alpha
PP2A	Protein phosphatase 2A
PROWESS	Recombinant Human Activated Protein C Worldwide Evaluation In Severe Sepsis
PS	Phosphatidylserine
PT	Prothrombin time
qPCR	Quantitative polymerase chain reaction
rhAPC	Recombinant human activated protein C
RIPK1	Receptor-interacting serine/threonine-protein kinase 1
RT-PCR	Reverse transcription polymerase chain reaction
RyR	Ryanodine receptor
SELE	E-Selectin
sEPCR	Soluble endothelial protein C receptor
SERCA	Sarcoplasmic/endoplasmic reticulum calcium ATPase
SIRS	Systemic inflammatory response syndrome
SOFA	Sequential Organ Failure Assessment score
TACE	Tumour necrosis factor alpha converting enzyme
TAFI	Thombin-activatable fibrinolysis inhibitor
TF	Tissue factor
TFPI	Tissue factor pathway inhibitor

TM	Thrombomodulin
TNF-α	Tumour necrosis factor-alpha
tPA	Tissue plasminogen activator
TTP	Time to peak
VCAM	Vascular cell adhesion molecule
VSMC	Vascular Smooth Muscle Cell
α_2M	Alpha-$_2$-Macroglobulin
α_1AT	Alpha-$_1$-antitrypsin

Chapter 1 General Introduction

1.1 Thrombin generation

Thrombin generation is characterized by the sequential activation of a series of serine proteases that can be broadly divided into initiation, propagation and amplification stages of coagulation [1]. Upon injury and consequent tissue factor exposure, the activation of factor VII leads to initial thrombin generation, which can then propagate further thrombin generation through activating components within the intrinsic pathway [2]. This leads to amplification in the coagulant process with the rapid and explosive conversion of fibrinogen to fibrin and the securing of haemostasis [3]. Simultaneously, there is thrombin-mediated platelet activation through protease activated receptors (PARs) [4] as well as thrombin cleavage of activated factor XIII (factor XIIIa) to stabilize fibrin formation [5],[6]. Further clot consolidation is mediated by thrombin through activation of thrombin-activatable fibrinolysis inhibitor (TAFI), which retards clot lysis by removing binding sites for the attachment of tissue plasminogen activator (tPA) to fibrin for consequent lysis [7]. Clot-based thrombin, however, can still organize and consolidate the fibrin structure, and also trigger the repair/healing mechanisms following injury[8]. Whilst a burst in thrombin generation is crucial in achieving prompt haemostasis, uncontrolled thrombin generation may be harmful. Indeed, thrombin generation is kept in close check under physiological conditions with a short half-life of only 15 seconds [9]. It is rapidly inactivated by different inhibitors within the family of serine protease inhibitors (SERPINS), such as antithrombin [10], α_1-antitrypsin (α_1AT) and heparin co-factor II [11], as well as by α_2-macroglobulin (α_2M) [12]. At the boundaries of tissue injury, any excess thrombin is then bound by the receptor TM on intact endothelial

surfaces. TM-bound thrombin can no longer express procoagulant activity ([13],[14] but instead activates PC [15].

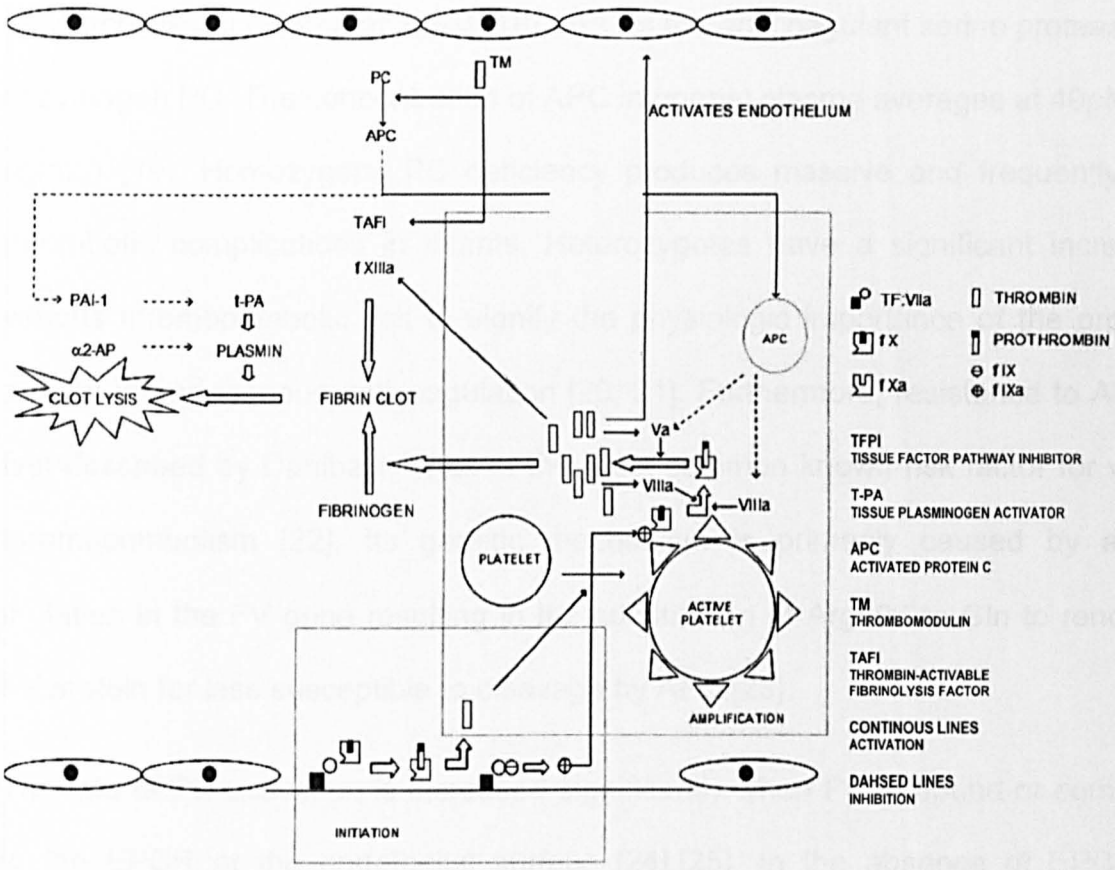

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Figure 1.1 Schematic representation of the main coagulant, anticoagulant and fibrinolytic systems in relation to the vascular endothelium (Modified from Amaral *et al,* 2004) [16]

1.2 Protein C Activation

PC is a vitamin K dependent glycoprotein like protein S, prothrombin, FVII, FIX and FX [17]. These are all synthesized in the liver. PC has a molecular weight of 62kDa and circulates in plasma at 70nM [18]. APC is the anticoagulant serine protease form of zymogen PC. The concentration of APC in normal plasma averages at 40pM (~2.3 ng/mL) [19]. Homozygous PC deficiency produces massive and frequently lethal thrombotic complications in infants. Heterozygotes have a significant increase in venous thromboembolic risk to signify the physiologic importance of the protein C system in endogenous anticoagulation [20, 21]. Furthermore, resistance to APC, as first described by Dahlback *et al.* is the most common known risk factor for venous thromboembolism [22]. Its genetic mechanism is primarily caused by a point mutation in the FV gene resulting in the substitution of Arg506 to Gln to render the FV protein far less susceptible to cleavage by APC [23].

The rate of PC activation is increased significantly when PC is bound or complexed to the EPCR at the endothelial surface [24],[25]. In the absence of EPCR, PC activation is reduced by 20-fold [26]. The resulting APC can then express anticoagulant properties by cleaving and inactivating factors Va [27],[28] and VIIIa [29] to reduce thrombin generation and procoagulant activity. As with other enzymes, APC has its own regulatory network consisting of PC inhibitor [30] and α_1AT as first-line inactivators [31] with α_2M as a secondary, calcium dependent inhibitor [32] [33]. Of interest is that APC anticoagulant activity in solution can be inhibited by soluble EPCR (sEPCR) [34]. This is somewhat paradoxical as membrane-bound EPCR promotes anticoagulant activity. This apparent Janus type function is due to altered exposure of the APC active site upon conformational change in EPCR, as a result of cleavage at the cell surface [35]. sEPCR, when cleaved and released from the cell

surface by tumour necrosis factor alpha converting enzyme (TACE) [36], is 4kDa smaller than its membrane counterpart. Receptor shedding is up regulated by thrombin [37],[38] and this apparent auto-regulatory mechanism of EPCR could theoretically modulate coagulant activity in the immediate vicinity of the cell surface. However, this alteration in APC within sEPCR could have other structure-function consequences given that it retains proteolytic activity towards small substrates [39]. Membrane-bound EPCR is homologous to the CD1/major histocompatibility class 1 family of molecules, most of which are involved in inflammation and antigen presentation [34]. Indeed the crystal structure of EPCR in complex with PC shares similarities with phospholipid binding within the antigen-presenting groove of the CD1 family [40]. Presentation and exchange of pathogen or cell-derived lipids may be involved in the processes by which these molecules modulate the inflammatory and immune response [41]. The relatively long half-life of APC, at approximately 20 minutes [42], maintains a circulating concentration of 38pmol/l [19]. This contrasts with the much shorter half-life of thrombin and is likely to relate to the importance of maintaining vascular patency following localization of clot formation. Thrombin needs to be rapidly inactivated in solution to ensure that its potent effects are not further disseminated.

1.3 EPCR

EPCR was discovered as a member of the PC pathway in 1994 by Fukudome *et al*, [43] Human APC and PC was found to bind to the endothelial cell surface selectively and saturably in a Ca^{2+} dependent manner with a dissociation constant (Kd) of 30nM [43]. Removal of the gamma-carboxyglutamic acid (Gla)-domains of PC resulted in loss of the binding affinity [44]. EPCR consists of 221 amino acids [45] and is glycosylated at four possible sites to give a molecular weight of 49kDa [46]. It was

initially thought to be present only on endothelial cells [43] but has now been found in other cell types like neutrophils, monocytes, eosinophils [47-49], brain endothelial cells [50] murine haematopoietic stem cells [51] and human vascular smooth cells [52]. EPCR appears to be essential for normal embryonic development and plays a key role in preventing thrombosis at the maternal embryonic interface [53]. EPCR (-/-) embryos die before embryonic day 10.5 and have evidence of increased fibrin deposition [53]. However, mice with low levels EPCR are able to develop, survive and reproduce normally [54]. In addition, when EPCR (δ/δ) deficient mice are challenged by endotoxin, changes in parameters like blood pressure, heart muscle damage, neutrophil infiltration in heart tissue demonstrate that EPCR plays an essential cardioprotective role after acute inflammatory challenge [55]. There appears to be a differential vascular distribution of EPCR and TM with EPCR preferentially expressed in large vessels and TM being more abundant in the microvasculature [56]. However, colocalization of EPCR and TM is essential for efficient activation of PC. EPCR has also been found in a soluble [57], truncated form in plasma with a molecular weight of 40kDa, as described above. It is cleaved by metalloprotease activity that can be induced by thrombin and other inflammatory mediators [58]. Soluble EPCR retains the ability to bind PC and APC but such binding blocks their phospholipid association and results in inhibition of the anticoagulant properties of APC. Soluble EPCR levels have been found to be increased in patients with sepsis and systemic lupus erythematosus [59]. Another soluble form of EPCR is in microparticulate form (see later). In contrast to sEPCR, microparticle (MP) bound EPCR retains its full-length membrane-bound form, which APC can bind to and continue to express proteolytic activity. This has also been found in the circulation of patients with sepsis [60].

EPCR shows sequence homology (~17%) [45] to the CD1/ major histocompatibility complex (MHC) class I superfamily, most members of which are involved in inflammation [43]. In the study of the crystal structure of EPCR the similarity of EPCR with the CD1/ MHC was further confirmed and it was demonstrated that the extraction of the phospholipid binding domain of PC led to the loss of PC and EPCR binding [61]. Its role in inflammation appears at the present time to be via the mediation of APC activity primarily (described later).

1.4 APC

1.4.1 APC– Anticoagulant Role

The process of APC generation is primarily at the endothelial cell surface involving action of the cellular receptors TM and EPCR[35], as aforementioned. Upon TM binding, thrombin's procoagulant exosite becomes unavailable and this leads to preferential cleavage of PC when bound to EPCR (Figure 1.3) [62]. PC activation in the absence of EPCR binding is reduced by 80% [35, 46] and this emphasises the importance of co-proximity at the cell surface. The irreversible inactivation of factors Va and VIIIa by APC is catalysed by cofactors, which include protein S, factor V, high-density lipoprotein, phosphatidylethanolamine (PE) and glycosphingolipids (e.g., glucosylceramide) [35, 63, 64]. APC also has profibrinolytic activity through binding to plasminogen activator inhibitor-1 (PAI-1) and suppressing its activity in inhibiting tPA. However, the concentrations required to achieve that may not be physiologically relevant and a more likely mechanism is through suppression of thrombin and its activation of the pro-carboxypeptidase TAFI. This activity is TM-dependent [65] and can occur in the fluid phase as well as on the cell surface.

1.4.2 APC- Anti-inflammatory and Anti-apoptotic Roles

Direct anti-inflammatory properties of APC have now been established and these are independent of the effect achieved via suppressing thrombin generation [66]. APC's anti-inflammatory properties have been described through its effects on endothelial cells, monocytes, macrophages, neutrophils and eosinophils. This is generally through alteration of the gene profile to decrease release of pro-inflammatory cytokines and enhancing the release of anti-inflammatory cytokines, which leads to diminution in the expression of adhesion molecules [67, 68]. The mechanism of action is varied and the broad classification, at least at the time of this Thesis submission, can be considered in terms of EPCR-dependence, which in turn can be further considered as via PAR-1 or not. This is also true for APC anti-apoptotic effects, which have been mainly described in endothelial cells. EPCR and PAR-1-independent actions of APC have also been described. One of the most relevant effects of APC on endothelial cells in relation to its anti-inflammatory properties is the decrease in the levels of the adhesion molecules ICAM-1, VCAM E-Selectin and thrombospondin-1 on previously activated endothelium with TNFα [67, 69]. APC anti-apoptotic effects have been observed *in vitro* whereby APC can inhibit staurosporine-induced apoptosis on EaHy926 cells, HUVEC and the kidney cell line HEK293 [67, 70]. Mediation of these cytoprotective effects are thought to occur mainly though APC-induced changes in the gene profile (described later). APC treatment has also been found to inhibit the expression of nuclear factor kappa beta (NFκβ). Firstly, there is a reduction in gene expression to decrease NFκβ mRNA levels on HUVEC. Secondly, APC suppresses the expression of p52, which together with p50, is one of the subunits that constitutes NFκβ [67]. In addition, APC inhibits the binding of NFκβ to target sites in the nucleus and the degradation of I-kappa Bα, which is a protein that together with I-kappa B β binds and

retains NFκβ in the cytoplasm. Furthermore, APC has been found to inhibit the binding of the activator protein-1 (AP-1) to target sites and the activation of the mitogen-activated protein kinase (MAPK) pathway [71]. p53, a tumour suppressor protein has been broadly reported to be affected by APC in leading to cytoprotective effects. This was found in HUVECs and in a hypoxic human brain endothelial cell model whereby APC inhibited p53 transcription [50,68,69]. This anti-apoptotic effect was independent from the above mentioned suppression of the gene expression of the apoptotic Bcl-2 homolog (A1) and inhibitor of apoptosis protein 1 (IAP-1) [50].

Riewald *et al* found that the cytoprotective effects of APC are dependent on the active site of APC, the binding of APC to EPCR and its signalling through PAR-1 [69, 70]. Mosnier *et al* and Riewald *et al* used the PAR-1 agonist peptide TFLLRNPNDK to prove that it could mediate cytoprotective properties in a similar fashion to APC [69, 72]. However, there has been an on-going debate over whether this is physiologically relevant as thrombin activation of PAR-1 generates a pro-inflammatory effect. The fact that APC is approximately 10^4 less potent in cleaving PAR-1 that thrombin [73] has raised controversy about how thrombin can induce pro-inflammatory effects whilst APC causes the exact opposite signal via the same receptor, PAR-1 [69]. Recently, a mechanism linking the activation of endogenous PC and the consequent APC activation of PAR-1 has been described and this is due to colocalisation of the critical receptors involved; i.e. PAR-1, EPCR and TM within lipid rafts on endothelial cells [74]. In addition, occupancy of EPCR by protein C/APC results in recruitment of PAR-1 onto a protective signalling pathway by its coupling to G_i-protein [74, 75]. Other studies have also demonstrated that APC-cleaved PAR-1 is retained on the membrane whereas thrombin-cleaved PAR-1 is internalised and degraded leading to a differential availability of PAR-1 on the cell surface [76, 77].

Overall, it seems that there is different trafficking in the signalling of APC and thrombin through PAR-1 to produce the two opposing effects on cells [76]. Further studies in animal models suggest that the APC-EPCR-PAR-1 mechanism is physiologically relevant [69, 78, 79]. In addition, the work from our group in Liverpool would suggest that this mechanism functions in humans given the demonstration of APC-EPCR-PAR-1-dependent MP release into the circulation of patients receiving recombinant APC (rhAPC) treatment [80].

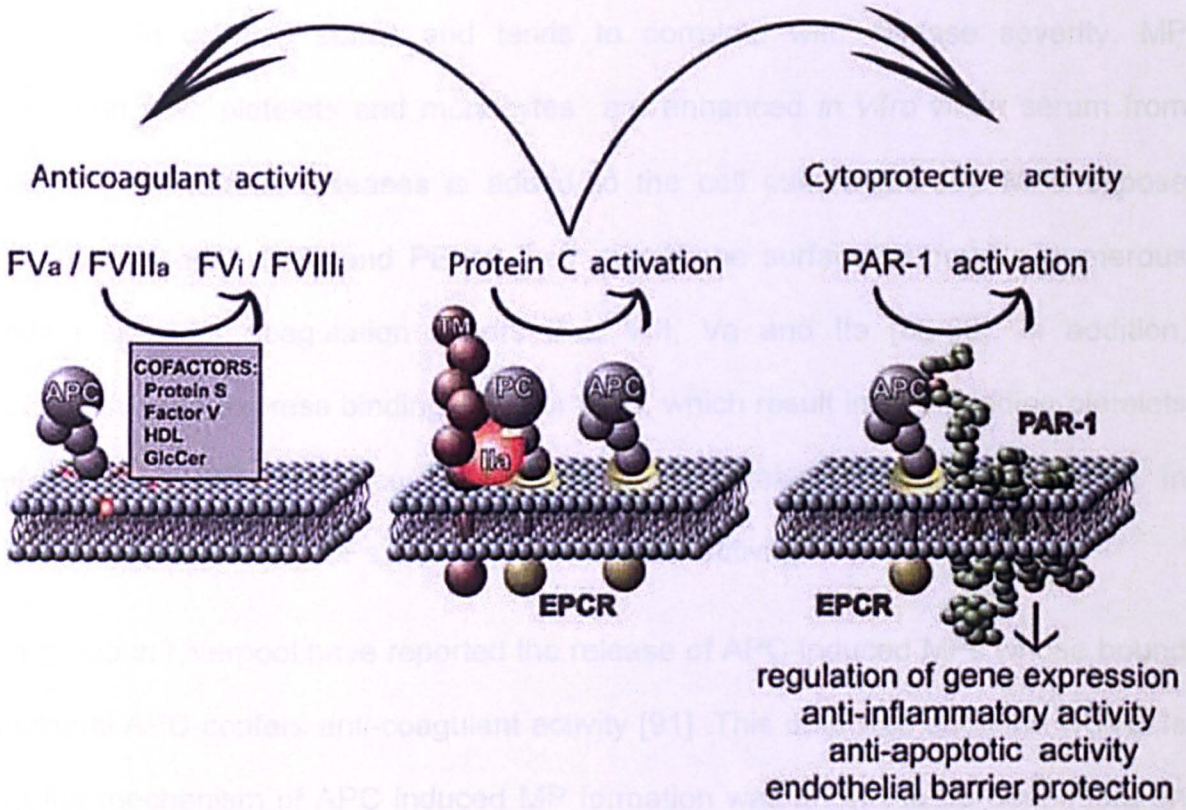

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Figure 1.2 Schematic model of APC's cytoprotective and anticoagulant activities (Mosnier and Griffin, 2006) [64].

EPCR localizes protein C on the endothelial cell membrane and enhances the activation of protein C. The anticoagulant activity of APC is inhibited when APC is in

complex with EPCR. EPCR acts as a cofactor for the direct effects of APC on cells and activation of PAR-1 by APC is EPCR-dependent. When APC dissociates from EPCR, it can express its anticoagulant activity, especially when bound to the surface of activated platelets or endothelial cells, various micro particles, or lipoproteins.

1.5 APC and Microparticles

MPs also play an important role in physiological coagulation [81, 82]. Their role has been studied in various diseases. In general, the total level of circulating MPs increases in disease states and tends to correlate with disease severity. MP production from platelets and monocytes are enhanced *in vitro* when serum from patients with various diseases is added to the cell culture [83-85]. MPs expose phosphatidylserine (PS) and PE on their membrane surface to provide numerous binding sites for coagulation factors IXa, VIII, Va and IIa [86-89]. In addition, endothelial MPs express binding sites for vWF, which result in MPs binding platelets to form stable MP-platelet conjugates that are detectable in the circulation [90]. In general, it appears that MPs induce pro-coagulant activity.

Our group in Liverpool have reported the release of APC-induced MPs whose bound functional APC confers anti-coagulant activity [91] .This data was obtained HUVECs and the mechanism of APC induced MP formation was shown to be dependent on the EPCR-APC-PAR-1 axis. As previously described (above) APC signalling requires the co-proximity of PAR-1-EPCR. Cheng *et al* (2003) showed that APC infusion in to mice causes PAR-1 dependent down regulation of pro-apoptotic p53 and neuroprotection in an ischaemic brain model. In this model however, only a fraction of PAR-1 colocalises with EPCR but the beneficial effects of APC were still observed at EPCR-deficient sites. Our previous data could explain this benefit with

APC induced MPs providing a means of circulating the EPCR-APC complex. We also know now that rhAPC treated septic patients have significantly higher levels of circulating EPCR-MPs [60]. As APC treated patients derive systemic anticoagulation from both free and MP-bound APC, a strategy that only delivers the EPCR-APC complex by bioparticles could be promising to decrease the risk of bleeding. Such results would have application to stroke and cardiovascular medicine where use of thrombolytic therapy increases haemorrhagic risk.

1.5.1 Detecting MPs

MPs play a role in coagulation, cell activation, inflammation and transport of biomarkers [92]. Therefore, the characterisation and quantification of MPs is of interest, especially in relation to translational medicine with its objective of directly improving patient care. MPs are generally studied from plasma as an indication of the *in vivo* state or from cell culture supernatants for more controlled characterisation. As early as 1946, Chargaff *et al.* observed that high speed centrifugation of plasma would lead to the prolongation in the clotting time of the sample. This led to the speculation that pro-coagulant particles in platelet free plasma (PFP) [93] exist that are precipitable and thereby removable through centrifugation. Wolf *et al.* showed that platelets release sudanophilic lipid-rich cell membrane-derived particles [94]. These were detected in PFP and the particles were found to be able to generate thrombin and were consequently referred to as "platelet dust". Wolf *et al.* also found a direct correlation between the platelet count and the levels of these particles [94]. Studies using electron microscopy analysis revealed that endothelial cells also release MPs. These were studied *in vitro* after stimulus with TNFα by Combes *et al.* in 1999 [84]. MPs were shown to express the same antigens as the cell membrane in both resting and activated conditions [84]. In addition, the presence of E-Selectin, αVβ3,

platelet/endothelial adhesion molecule 1 (PECAM-1) and ICAM-1 on the surface of endothelial MPs implies their adhesive potential [84]. MPs can therefore be described as intact vesicles that are shed from the cell membrane and can vary in size from 0.1-1.0μm [81, 82]. MPs lack nuclei but contain membrane structures, components of the cytoplasm and specific antigens from the cell of origin. MPs can be produced spontaneously of after activation or apoptosis [81, 82, 95-97].

1.5.2 MP Interaction with other Cells

The interaction of MPs with cells has been broadly reported. Boulanger *et al.* showed that MPs from patients affected by myocardial infarction could alter the usual relaxation of the aortic vein ring after stimulation with acetylcholine. This effect was endothelium-dependent and implied that MPs from patients with myocardial infarction contributed to endothelial vasomotor dysfunction [98]. Amabile *et al.* reported that endothelial MPs from patients with end-stage renal failure contributed to endothelial dysfunction through the impairment of endothelium-dependent relaxation and cyclic guanosine monophosphate generation [99]. The effect of platelet-derived MPs on the vascular tone was reported by Pfister *et al.* MPs from platelets were found to carry arachidonic acid on their surface and contribute to the synthesis of additional thromboxane when associated with endothelium. Consequently, platelet MPs would increase arachidonic acid-induced contractions in the aorta [100].

Distler *et al.* reported the release of MPs from Jurkat T cells after induction of apoptosis. These MPs were also rapidly cleared by macrophages and those macrophages which had taken up T-cell-derived MPs underwent apoptosis as analysed by binding of annexin V, caspase-3 activity and additional release of MPs

[101]. In summary, these studies demonstrate that MPs of different origins have interactions with different cell types and are able to impart a physiological effect on these cells.

1.6 APC and the Endothelium

Although the role of APC in modulating microvascular coagulation through the inhibition of thrombin generation had been recognized, any direct effects of APC, independent of its anticoagulant effect on endothelial function had not been studied. Using transcriptional profiling and cluster analysis it was demonstrated that rhAPC altered gene expression within cell signalling pathways of inflammation and apoptosis [102]. HUVECs were treated with APC (8-183nM) and TNFα (1ng/ml). For the microarray experiments, the cells were treated with APC for 16 hours and TNFα for the last 7 hours. Transcript profiling showed that there were a number of genes regulated by either APC or TNFα alone. TNFα activated genes known to be associated with pro-inflammatory and proapoptotic pathways were found to be counter modulated by APC treatment. In particular, NFkB expression was suppressed by both APC alone and in combination with TNFα treatment (shown using semi-quantitative RT-PCR). rhAPC also suppressed ICAM-1, VCAM-1 and E-Selectin expression induced by TNFα (shown using FACS).

Pro-apoptotic genes such as calreticulin were suppressed by rhAPC, whereas anti-apoptotic markers such as A1, IAP and proliferating cell nuclear antigen (PCNA) were increased. This was translated in to a reduced number of cells undergoing apoptosis in response to staurosporine. This has relevance for growing evidence for the role of apoptosis in the systemic inflammatory response and sepsis [103-105].

This study provided mechanistic understanding for the then recent data supporting the potential therapeutic use of APC in sepsis.

Similar to our work, Franscini *et al,* studied the gene expression profiling in HCAECs exposed to pro-inflammatory stimuli and the influence of APC on expression [68]. This study was the first to use a primary human microvascular endothelial cell system to study the effects of rhAPC on gene expression. Cytokine mixtures were used to represent mild to severe inflammatory conditions. 90% confluent cells were used, passage p3-6 in T75 flasks or 6 well plates. A cocktail of rhIL, rhTNFα and rhIFN gamma in media were used to represent the proinflammatory stimulus. Gene expression profiling was recorded following 6 hours of treatment with the cytokine mixture and compared to unstimulated cells. For genes of interest confirmation of regulation was confirmed by RT-PCR. Western blotting and cytokine ELISAs were used to demonstrate the protein. Of 8400 genes analysed, 85% of genes were induced, 15% suppressed. The highest numbers of regulated genes were found in the functional clusters for adhesion, transcription factors, cell signalling, intracellular transducers and metabolism. The effect of rhAPC was then studied with respect to specifically ICAM-1, VCAM-1, IL-6, IL-8, MCP-1 and NFKB. 5ug/ml APC was used with the HCAECS and cytokines for 24 hours total. Down regulation of expression was found more significantly with the milder pro-inflammatory combinations. The authors felt that using a combined cytokine mixture better mimicked the pathophysiology of systemic inflammation. It was further highlighted that some genes may not be caught by the profiling time frame.

Wu *et al* [106], compared changes in endothelial cell gene expression profiling in response to thrombin, TNFα and lipopolysacharide (LPS), in particular focusing on 5 target genes including ICAM-1 and their kinetics of activation and downstream

signalling pathways. Human pulmonary Artery Endothelial Cells (HPAECs) were used to determine the effect of the different inflammatory mediators on target gene expression. Comparing results for ICAM-1, TNFα induced a 4.4 fold induction of ICAM-1 mRNA with maximum levels at a concentration of 2.5ng/ml at 2 hours. The effect of LPS was much lower. Compared with endothelial cells, using vascular smooth muscle cells (VSMCs) TNFα resulted in a 3.5 fold induction of ICAM-1 mRNA with maximum levels at 2.5-10ng/ml at 1-2 hours. LPS induced a 2.5 fold induction with maximum levels at <100ng/ml at 1-2hours.

1.7 APC and Sepsis

Sepsis has a major health impact in society, both in well-resourced and under-resourced settings. In the United States of America (USA), 751,000 cases of severe sepsis occur every year [107] and in the United Kingdom (UK), 30% of the patients in intensive care unit (ICU) suffer severe sepsis during their stay [108]. One of the prime difficulties is in its diagnosis. Sepsis has had several definitions due to its complexity and is best considered as the host response to infection. The term of systemic inflammatory response syndrome (SIRS) was established in 1991 by the North American Consensus Conference [109] to differentiate non-infective causes of inflammation but is considered by many ICU clinicians as an oversimplification, especially in the assessment of critically ill ventilated patients. One of the latest definitions of sepsis was agreed in 2001 during a consensus conference under the guidance of the Society of Critical Care Medicine, the European Society of Intensive Care Medicine, the American College of Chest Physicians and the Surgical Infection Societies [110]. This definition categorises sepsis as the clinical syndrome characterised by the presence of both infection by a pathogenic microorganism of a cavity or usually sterile tissue and a systemic inflammatory response. Severe sepsis

is then characterised by sepsis with organ dysfunction complications and septic shock occurs when there is also persistent arterial hypotension unexplained by other causes and despite resuscitation [110]. The somewhat ambiguous and long list of signs indicative of sepsis invariably leads into difficulty for an early diagnosis of sepsis and various specific markers have been proposed to overcome this. Examples of this markers are C-reactive protein (CRP) or procalcitonin [111, 112], cytokines [113, 114], endotoxin levels [115] and the biphasic activated partial thromboplastin time (aPTT) waveform [116, 117]. Although these markers have been found to be useful, none are fully specific for sepsis. Therefore, the use of a combination of these markers has been considered to be potentially more accurate for the diagnosis of sepsis than one marker alone [118]. Whilst this may be theoretically advantageous, this adds to practical complexity that is not suited for the acute pace of the ICU setting.

Acquired PC deficiency is a well-recognized consequence of sepsis and studies have demonstrated its value as a prognostic marker [119]. There is a negative correlation between PC levels in septic patients with increasing morbidity and mortality [120]. This is due to endotoxin and cytokine-mediated down regulation as well as enzymatic cleavage to release the soluble receptors [121], [122], [123]. In this situation, the procoagulant and pro-inflammatory effects of thrombin predominate, resulting in microvascular thrombosis and vascular leakage [124].

Therapeutically, administration of a recombinant form of human APC had been shown to significantly reduce the relative risk of death in patients with severe sepsis. A randomized, double blind, placebo controlled, multicentre trial [The Recombinant Human APC Worldwide Evaluation in Severe Sepsis (PROWESS) trial] demonstrated a 19.4% reduction in the relative risk of death from any cause at 28d

for patients with a clinical diagnosis of severe sepsis receiving recombinant APC [125]. Patients who demonstrated most benefit from the drug were those with the highest Acute Physiology and Chronic Health Evaluation II (APACHE II) scores who had extensive organ dysfunction [126]. To improve on the benefit-risk ratio of APC treatment in sepsis, knowledge of the mechanisms involved *in vivo* would be highly relevant. Neutralizing thrombin is likely to be an important aspect, but failures of treatment with high-dose antithrombin [127] or tissue factor pathway inhibitor (TFPI) [128] suggest that the success of APC is due to properties other than anticoagulation.

1.8 Coagulation and Inflammation in Sepsis

1.8.1 Inflammation-Induced Coagulation

A pivotal factor in inflammation-induced coagulation problems of sepsis is tissue factor (TF) expression [129]. TF is a 45 kDa transmembrane protein that is constitutively expressed in different cells in the body, which are usually within tissues that are not in direct contact with blood [130]. Under normal conditions, the endothelium acts as an anti-thrombotic surface [131]. While TF is the major initiator of coagulation, endotoxin, foreign bodies and negatively charged particles can also initiate coagulation via contact system activation and the intrinsic coagulation pathway [16]. TF appears in blood if vascular damage has occurred or if blood cells present it on their surfaces. In severe sepsis, mononuclear circulating cells express TF due to their induction by pro-inflammatory cytokines that is considered to be mainly IL-6 [132, 133]. TF binds to factor VIIa and this complex catalyses the activation of factor X and factor XI [134]. Factor Xa combined with factor Va, prothrombin (factor II) and calcium in the presence of anionic phospholipids

constitutes the prothrombinase complex, which catalyses the generation of thrombin (factor IIa) [135, 136]. A prime action of thrombin, especially if generated as an explosive burst, is the conversion of fibrinogen into fibrin [137]. As a mechanism to amplify thrombin generation, the back-activation of factor XI by the thrombin-factor Va complex will generate larger amounts of factor Xa. Secondly, the activation of cofactors V and VIII by thrombin contributes to this positive amplification of the coagulant process [138]. In addition, the activation of factor XI by thrombin leads to additional activation of factors IX and X to drive the pro-coagulant response [129]. Thrombin activates factor XIII to stabilise the fibrin clot by creating cross-links that makes the clot more resistant to degradation by plasmin [139, 140]. Thrombin also activates TAFI to retard clot lysis [7]. In relation to platelets, thrombin produces their activation that contributes to the fibrin clot and the release of their procoagulant content. Activated platelets express P-Selectin to enhance endothelial cell-leukocyte adhesion whilst also inducing the expression of TF on monocytes [134, 141] to further enhance the coagulant response. Whilst nucleated cells and coagulant factors are important in these processes, the assembly of tenase complexes, constituted by factors X, IXa, VIIIa and calcium or the prothrombinase complex, as mentioned above, is greatly facilitated by anionic phospholipid surfaces that are largely provided by activated platelets [141, 142].

In inflammatory conditions, TF can also be induced in the circulatory system by CRP, advanced glycosylated-end products and cytokines, like TNF-α and IL-1β [143, 144]. Usually, this inducible TF is expressed on macrophages and monocytes [145]. In addition, endothelial cells also express TF on their surface after stimulation with cytokines *in vitro* [143, 144].

Overall, sepsis results in the widespread and systemic activation of coagulation to cause the formation of intravascular fibrin, resulting on thrombosis on small and midsize vessels, which can lead on to development of disseminated intravascular coagulation (DIC). This can compromise the supply of blood to vital organs and DIC can be regarded as a major causal factor in multi-organ dysfunction [146]. In biopsies taken from patients with meningococcal septicaemia, there is down regulation in the expression of both TM and EPCR with evidence of dysfunctional PC activation [147].

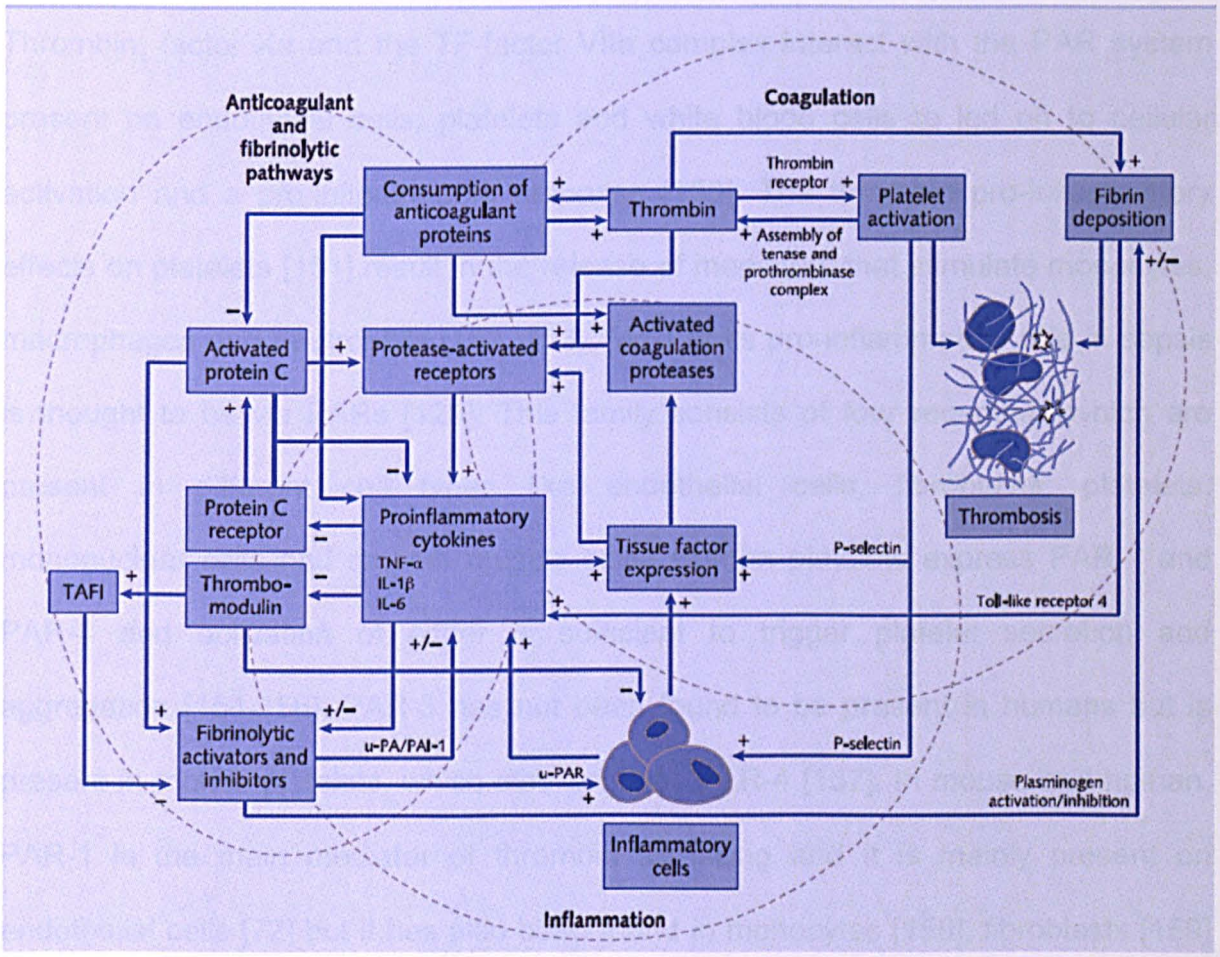

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Figure 1.3 Schematic overview of the major crosstalk pathways interrelating coagulation and inflammation (Levi and van der Poll, 2004) [148]

+: stimulatory effect; –: inhibitory effect; IL: interleukin; PAI-1: plasminogen activator inhibitor-1; TAFI: thrombin activatable fibrinolysis inhibitor; TNF: tumour necrosis factor; u-PA: urinary plasminogen activator; u-PAR: urokinase-type plasminogen activator receptor

1.8.2 Coagulation-Induced Inflammation

It is becoming very clear that components of the coagulation system contribute to the inflammatory process in sepsis. In mirroring inflammation-induced coagulation, coagulation also triggers the pro-inflammatory process in sepsis [129, 149]. Thrombin, factor Xa and the TF-factor VIIa complex interact with the PAR system present on endothelial cells, platelets and white blood cells to led on to cellular activation and a pro-inflammatory response [150]. The thrombin pro-inflammatory effects on platelets [151] result in the release of mediators that stimulate monocytes, macrophages and neutrophils [152, 153]. Most of its pro-inflammatory role in sepsis is thought to be via PARs [129]. This family consists of four receptors, which are present in different cell types like endothelial cells, fibroblasts, platelets, mononuclear cells and smooth muscle cells. Human platelets express PAR-1 and PAR-4 and activation of either is sufficient to trigger platelet secretion and aggregation [154-156]. PAR-3 has not been found to be present in humans but is present in mouse platelets, which also contains PAR-4 [157]. In mouse and human, PAR-1 is the main mediator of thrombin signalling and it is mainly present on endothelial cells [72] but it has also been found in monocytes [158], fibroblasts [159] and smooth muscle cells [160]. Fibroblasts and endothelial cells also express PAR-2 [161, 162]. Their activation occurs by proteolysis and exposure of a neo-N-terminus that consequently acts as a ligand leading into intracellular signalling [163]. Thrombin can cleave PARs 1, 3 and 4. PAR-2 can be cleaved by trypsin. PAR-1 can also be

the receptor for TF-factor VIIa complex and factor Xa [129, 164]. Binding of PAR-2 by TF-factor VIIa complex results in up regulation of pro-inflammatory responses on macrophages by increasing the production of adhesion molecules and production of reactive oxygen species as well as TNFα and IL-6 [165].

The cellular receptor TM also plays an important role in the crosstalk between coagulation and inflammation in sepsis. In addition to reducing thrombin availability and activity [66], its lectin domain is essential for regulating the inflammatory response. An endotoxemic mouse model lacking the lectin-like domain of TM showed increased recruitment of neutrophils to the lungs and reduced survival. In addition, lectin-TM deficiency did not impair the activation of protein C, indicating that the anti-inflammatory effect of the lectin domain of TM is APC activation independent [166].

1.9 The Emergence of APC as a Clinical Agent

Increasing understanding of the protein C network and its functional overlap between coagulation and inflammation prompted further investigation in to the potential role of protein C as a treatment for sepsis.

1.9.1 Pre-Clinical Studies

In 1977 it was shown by Fletcher *et al.* that the extra-corporeal canine circulation could be maintained in the absence of heparin [167]. Later it was further found using this model that dogs were protected when the pump was run for thirty minutes and then shut off just prior to injection with LD100 E. Coli endotoxin. This suggested that the blood had acquired the capacity to provide protection via the pump and complemented work by Esmon *et al.* in 1982 [15] showing that thrombin infused into the coronary circulation generated APC thorough interaction with endogenous TM.

Infusion of thrombin with endotoxin was also protective further supporting the suspicion that the generation of APC was key [168].

Experimental models of sepsis have helped delineate the relevance of the thrombin–APC axis. Firstly, a recently developed mouse model of the pro-thrombotic FV Leiden mutation has shown that heterozygote animals are protected against the lethal effects of sepsis, whilst homozygotes have an adverse outcome. This is borne out in humans, with a survival advantage in sepsis for patients who are heterozygous for the FV Leiden mutation [169]. The implication is that enhanced, but not excessive, thrombin generation will optimize APC generation, which provides protection against the effects of sepsis.

Histological changes from the EPCR blocked animals showed increased microvascular thrombosis, widespread necrotic foci and white cell infiltration within the adrenals, kidneys and liver. In separate experiments, heart muscle damage was more severe in EPCR-deficient mice as compared to wild-type after endotoxin administration [170].

Similar studies were then also performed in primates demonstrating the prophylactic and therapeutic efficacy of APC and recombinant APC [171, 172] without causing severe bleeding complications. Exogenously added APC prevented the coagulopathic response and lethal effects of LD100 concentrations of E. Coli in the baboon, and subsequently it was demonstrated that blocking protein C activation led to a more severe pathological response which could be prevented by co-infusion with APC [173]. Although administration of APC was protective it should be noted that relatively high concentrations were required questioning whether APC played a natural role in the defence against gram-negative sepsis.

1.9.2 Clinical Studies

Phase II

A phase II study by Eli Lily showed that following a four day treatment course of APC 24ug/kg/h, levels of the haemostatic marker d-dimer and inflammatory marker IL-6 were reduced. Reduction in these biomarkers associated with severe sepsis coincided with an apparent 40% improvement in survival rate [174].

Phase III

These observations led to the phase III protein C Worldwide Evaluation in Severe Sepsis (Prowess Trial), randomized, double blind and placebo controlled[125]. The incidence of serious bleeding during the drug infusion in Prowess was 3.5% vs. 2.0% for Drotecogin alfa (DrotAA) and placebo groups, respectively. In both groups, serious bleeding occurred primarily in patients already with a haemorrhagic predisposition such as GI ulceration, traumatic injury, invasive procedures, platelet counts <30.

Of interest the baseline levels of some of the biomarkers measure showed significant and clinically meaningful trends relative to disease severity. The four most informative were d-dimer and prothrombin time (PT) with elevated levels in 99.7 and 93.4% of patients and those of protein C and anti-thrombin with decreased baseline levels in 88 and 82% of patients [175]. The latter were more reduced amongst patients in the higher APACHE quartiles indicating a correlation between the abnormality and severity of clinical presentation [176].

Phase IIIB trials

The Extended Evaluation of Recombinant Human APC (ENHANCE) Trial was commenced in 2001 with the objectives of obtaining more safety and efficacy data on the use of DrotAA in patients with severe sepsis [177]. This was an open label, single arm trial and unlike in PROWESS DrotAA infusion could be initiated up to 48 hours after the first sepsis induced organ dysfunction. The baseline enrolled population was more severely ill however efficacy results were similar to PROWESS in terms of 28 day mortality and survival curve. The incidence of serious bleeding was higher (3.6% vs. 2.4) and there was a greater incidence of ICH. An important finding in this study was the higher survival rate for patients treated within the first 24 hours of organ dysfunction over those treated later.

Administration of Drotecogin alfa in early stage Severe Sepsis (ADDRESS) was a double blind, placebo controlled randomized trial instructed by the FDA to study adults with severe sepsis at low risk of death[178]. The sample size was small and subgroups were not adequately powered making it difficult to draw meaningful conclusion from or compare with PROWESS.

APC was approved by the US FDA in November 2001 for the treatment of adult patients with severe sepsis at high risk of death. In 2002 it was recommended by the European Agency for the Evaluation of Medical Products that use be restricted to patients with two or more sepsis induced organ dysfunctions.

With this focus on the APC anti-inflammatory role involving PAR-1, mutants have now been created that are less anti-coagulant but retain anti-inflammatory effects [179]. The clinical relevance of these molecules awaits further investigation.

1.10 APC and its Role on Other Organs

1.10.1 Brain

Plasma protein C is an inverse risk factor for ischaemic stroke [180]. Low levels of circulating APC or resistance to its effect are potential risk factors for stroke [181, 182]. Following an ischaemic stroke, infiltration of neutrophils to the site potentiates ischaemic neuronal injury [183]. Signals generated from the binding of APC to EPCR with subsequent cleavage of PAR-1 translate into anti-apoptotic and endothelial barrier-protective effects, which have been shown to be physiologically relevant in experimental models of ischemic stroke

Shibata *et al* used a murine model of focal ischaemia to look at the anti-thrombotic effects in stroke in addition to the potential to control the leukocyte response post brain injury [184].

APC 2mg/kg was administered 15 minutes before or 10 minutes after middle cerebral artery (MCA) occlusion. Pre-treatment with 2mg/kg resulted in a 25% improvement in cerebral blood flow ($p=0.05$), reduction in brain infarct volume by 59% ($p<0.02$) and oedema volume by 50% ($p<0.05$) and complete elimination of neutrophils from brain tissue and ischaemic vessels. APC 2mg/kg given 10 minutes after MCA occlusion also showed protective effects. APC reduced the number and intensity of ICAM-1 positive blood cells preventing adhesion to the vessel wall and transport across the blood brain barrier. APC has been shown to protect against ischaemia/ reperfusion renal damage [185] and endotoxin induced vascular pulmonary injury [186] by inhibiting leukocyte activation, Attenuation of ischaemic brain damage by eliminating the leukocyte response was therefore felt probable.

Using an *in vitro* model of hypoxia induced apoptosis of human brain endothelial cells, Griffin *et al*, showed that PAR-1 and EPCR were required for APC to exert its anti-apoptotic effects [187]. APC blunted hypoxia induced increases in p53 mRNA and protein, Bcl-2 and reduced the pro-apoptotic Bax. Because the doses of recombinant murine APC needed to provide neuroprotection are low, unwanted anticoagulant effects are not observed making APC an attractive therapy for stroke.

tPa is the only US FDA approved therapy for ischaemic stroke and needs to be used within 3 hours of an acute event but carries a risk of cerebral haemorrhage and neurotoxicity [188]. Liu et al proposed that APC and tPA combination therapy may enhance reperfusion and ameliorate neurotoxicity [189]. They reported that APC reduced tPa induced cytotoxicity in cell culture and decreased infarction and neurological deficit. It was suggested that APC improves perfusion through binding to PAI1 and reduces inflammation through down-regulation of adhesion molecules such as VCAM-1/ICAM-1.

The induction of a potent $[Ca^{2+}]_i$ signal in brain endothelium by APC through PAR-1 was demonstrated by [190]. Furthermore this was dependent upon APC-EPCR binding. 50nM APC administered to brain endothelial cells (BECs) for 60 seconds elevated $[Ca^{2+}]_i$ from 83.1 nM to 381.7nM. Active site mutant APC or zymogen protein C did not produce a $[Ca^{2+}]_i$ response. The APC evoked rise was dose dependent (0.02-100nM) and BEC pre-incubation with anti-PAR-1 antibody completely inhibited the $[Ca^{2+}]_i$ rise. Using an antibody raised against the PAR2 clevage site did not block the APC induced $[Ca^{2+}]_i$ signal.

1.10.2 Gastro-Intestinal Tract

The mucosal microvasculature has been shown to be involved in intestinal inflammation [191]. Because of the higher ratio of endothelial surface to blood volume in smaller vessels, there is a relative increase in TM concentrations compared with the microvasculature and therefore greater conversion of PC to APC [35, 192, 193]. In inflammatory bowel disease (IBD) it is activated by aberrant CAM expression, cytokine production and angiogenesis. During inflammation however, the anti-inflammatory components of the PC pathway are down-regulated as previously described and observed in various models of experimental inflammation [194, 195]. Scaldaferri *et al* looked at the expression and function of the PC pathway players, their potential involvement in the pathogenesis of IBD and thus therapeutic possibilities with respect to intestinal inflammation[196]. Histological specimens from human surgical specimens with normal bowel mucosa showed strong immunohistochemical staining for TM and EPCR, however this was significantly reduced in active IBD but not non-inflamed IBD. The effect of TNFα on expression levels in human intestinal mucosal microvascular endothelial cells (HIMECs) was studied using 50ng/ml and showed significant reduction. In addition to suppressing TM and EPCR at the transcriptional level, TNF also increased soluble EPCR release supporting shedding of the receptor during inflammatory conditions. HIMECs were then treated with TNF in the absence or presence of 80nM human recombinant APC. APC inhibited TN alpha induced up regulation of the CAMs and other chemokines.

Using an *in vivo* murine model, healthy mice versus induced colitic mice were studied for any potential therapeutic effect of APC (0.1 and 1mg/kg). APC treated mice displayed less severe colitis demonstrated by less severe weight loss at the end of ten days treatment, an improved disease activity index and reduced

inflammation by histological assessment. There were no bleeding events in the animals.

1.10.3 Heart

Although there have been no studies looking at the intrinsic effect of APC on cardiac cells, some work has been performed exploring the anti-inflammatory properties of APC in addition to effects of left ventricular performance parameters [197]. Instrumented rats were pre-treated with APC before receiving E. Coli endotoxin. Measurements were taken 4 hours later to study the effect of APC treatment on cardiovascular function and inflammation. Myocardial contractile function was studied using a modified Langendorff isolated heart preparation containing a water-filled latex balloon inserted in to the left ventricle cavity and connected to a pressure transducer. LPS endotoxin alone induced significant reductions in LV systolic performance, however this was prevented in APC treated rats. APC also reduced LPS-induced drops in mean arterial pressure (MAP). No effect of APC was observed for either of these variables in control rats. Coronary perfusion pressure was measured as a derivative of the left ventricle end diastolic pressure, however in the absence of any changes it was suggested that APC affects intrinsic heart contractility independent of changes in coronary haemodynamics. APC also attenuated endotoxin induced inflammatory responses as represented by plasma levels of TNFα, nitrite/nitrate and endothelial cell leucocyte activation. As previously described some of these agents have been implicated in the pathophysiology of myocardial depression in sepsis, therefore overlap may exist in mechanisms contributing to myocardial function improvement. Bleeding complications were not observed with APC treated rats, however limitations with this study included the lack of any molecular detail, APC blocking experiments or graphical pressure volume curves.

Nacira *et al* used a similar endotoxic shock model recording changes 2 hours post LPS [198]. In addition to the mean arterial pressure, myocardial function was assessed by means of transthoracic echocardiography pre and post endotoxin to measure left ventricular ejection fraction. After 240 minutes prior to killing the rat, the heart was rapidly excised and frozen for further measurements of adenine nucleotide and lactate/pyruvate ratio measurements, morphologic analysis of leucocyte infiltration, MPO and MMP-9 activity.

Again APC improved the LPS-induced decrease in MAP. Reductions in left ventricle ejection fraction (LVEF), stroke volume and cardiac output as measured by ECHO were blunted by APC however these effects were not statistically significant. An APC-induced decrease in aortic iNOS expression and NO content was demonstrated however it was acknowledged that although iNOS restores vascular tone, it does not restore myocyte contractility. Limitations of the study proposed by the authors included the use of a rat model to represent clinical sepsis in humans and the use of APC concomitantly with LPS as opposed to post treatment.

1.11 Cardiac Dysfunction in Sepsis – The Mechanisms

Waisbren was the first to describe cardiovascular dysfunction due to sepsis in 1951 [199]. The precise cause of cardiovascular dysfunction in sepsis however still remains unclear and continues to be the focus of clinical and basic research [200].

2 stages of sepsis are recognized in relation to the progression of the condition. During the earlier stages, patients are hyperdynamic with bounding pulses, fever and hypotension. With increasing severity subjects become clammy, pale and hypotensive with low volume pulses. Progression to the latter state may be avoided

with adequate volume resuscitation, now demonstrated to be one of the most effective support therapies for sepsis [201].

A reduction in LVEF however can still be seen however in patients who are adequately volume replaced and this was first shown using portable radionuclide cineangiography by Calvin *et al.* in 1981 [202]. This has been confirmed more recently by echocardiographic assessment in septic patients [203, 204]. The relative contribution of the right ventricle to septic cardiomyopathy is less clear.

The mechanisms underlying myocardial dysfunction in sepsis are likely multifactorial and a number of agents/pathways have been implicated in the underlying process.

1.11.1 Global Ischaemia

An early hypothesis, however septic patients often demonstrate high coronary blood flow [205]. Studies in patients and animals confirm metabolic alterations in the septic myocardium however there is no overwhelming evidence supporting global ischaemia as the underlying cause of myocardial dysfunction in sepsis.

Septic patients may however have coexistent undiagnosed coronary artery disease (CAD), or infarction secondary to CAD both of which may be aggravated by the inflammation and hyper-coagulation associated with sepsis.

1.11.2 Myocardial Depressants

The theory of circulation myocardial depressant factors has been around for more than 50 years. Parillo *et al.* quantitatively linked the clinical degree of septic myocardial dysfunction with the effect of patients' serum on rat cardiomyocytes, where clinical severity correlated well with a reduction in myocyte shortening [206]. These effects were not seen using the serum of critically ill non septic patients.

Candidates for myocardial depressant substances include; lysozyme C, NO, cytokines and prostanoids (see below).

1.11.3 Cytokines

LPS is an obligatory component of gram negative bacterial cell walls and infusion of LPS induces haemodynamic effects seen in sepsis however it is felt that this agent alone is unlikely responsible for the spectrum of consequence that follows [207].

TNFα appears to be an early mediator of endotoxin induced septic shock [208]. Derived from macrophages but studies have also shown that TNFα is secreted by myocytes in response to sepsis [209]. Although application of anti- TNFα antibodies improves left ventricular function in patients with septic shock [210], monoclonal antibodies have failed to improve patient survival [211].

IL-1 depresses cardiac contractility through stimulation of NOS [212] however evaluation of recombinant IL-1-ra in phase III clinical trials failed to show any significant survival benefit [213].

1.11.4 Prostanoids

Elevated levels of prostanoids such as thromboxane and prostacyclin have been demonstrated in septic patients with the potential to alter coronary autoregulation and coronary endothelial function [214]. A clinical study looking at the effect of brufen versus placebo in septic patients unfortunately showed no survival benefit in the treatment arm [215].

1.11.5 Endothelin

Up regulation of endothelin 1 has been demonstrated within 6 hours of LPS [216] induced septic shock and over expression triggers an increase in cytokines such as TNF, IL-1, IL-6 leading heart failure and death [217]. The therapeutic use of ET-1 receptor antagonists is under evaluation with tezosentan, a dual endothelin A and B receptor antagonist showing improved cardiac index and left ventricular stroke work index in endotoxemic shock [218].

1.11.6 Nitric Oxide

NO has multiple biological effects on the cardiovascular system [219]. Low dose NO can increase LV function whereas higher does have been shown to induce dysfunction by depressing myocardial energy generation. Sepsis leads to the expression of inducible NOS (iNOS) in the myocardium, followed by high level NO production contributing to myocardial dysfunction through generation of cytotoxic peroxynitrite [220]. In septic patients the infusion of methylene blue (a nonspecific NOS inhibitor) improves mean arterial pressure and stroke volume reducing the requirement for inotropic support however does not alter outcome [221].

1.11.7 Adhesion Molecules

The up regulation of surface ICAM-1 and VCAM-1 in response to TNF/LPS has been shown in murine coronary endothelium and cardiomyocytes. Furthermore, antibody blockade of both is associated with reduced myocardial dysfunction [222].

1.11.8 Protein Phosphatases

It is reported that 16-18hrs post LPS injection, endotoxaemic rat hearts have increased cTnI phosphorylation at Ser23/24 residues that mediate protein kinase A

(PKA) dependent reduction of myofilament Ca^{2+} sensitivity [223]. With no reduction in calcium transient, reduced myofilament sensitivity was thought to underlie the contractile dysfunction. Further studies since have confirmed the importance of increased cTnI phosphorylation in sepsis by demonstrating that mice with cardiac specific replacement of cTnI with slow skeletal TnI lacking the PKA phosphorylation sites, are significantly protected against LPS induced cardiac dysfunction [224]. This protection was also observed in triton-skinned cardiomyocytes. Increases in cTnI phosphorylation could result from kinase activation or inhibition of dephosphorylation pathways. Protein phosphatase 2A (PP2A) is a major dephosphorylating enzyme which dephosphorylates both cTnI and phospholamban (Liu Q Am J Phys 2002. It is thought to have a major role in maintaining normal cardiac function as inhibition results in increased phosphorylation of cTnI and phospholamban [225] and transgenic mice over-expressing PP2A demonstrated reduced contractility [226]. *Marshall et al* investigated whether changes in the regulation of PP2A were responsible for sustained phosphorylation of cTnI in myocytes from endotoxaemic hearts [227]. Depression of cardiac function in septic myocytes, namely shortening, was reversed using a PKA inhibitor and/or a PP2A agonist. Both of these treatments were independent of changes in the calcium transient suggesting that the improvement in contractile function reflected a restoration in myofilament calcium sensitivity.

1.12 Cardiac Dysfunction in Sepsis - The Relevance of APC

Currently, there are no strategies to specifically target myocardial dysfunction in sepsis and APC use is limited by concerns over severe bleeding complications from systemic anti-coagulation.[228] A report by Dhainaut *et al* studied the Prowess data on efficacy and safety of APC treatment in patients with multiple organ dysfunction

[229]. The landmark Prowess Trial demonstrated a significant reduction in 28 day all-cause mortality (19.4% relative risk reduction p=0.005) and morbidity for the treatment group[125]. APC was subsequently approved for patients with severe sepsis with multiple organ failure (2 or more dysfunctions based on Prowess entry criteria). The benefit-risk profile was better for such patients versus those with single organ dysfunction. Organs assessed were cardiovascular, respiratory, haematology, renal and metabolic dysfunction. Cardiovascular dysfunction criteria were either of the following; arterial systolic blood pressure < 90mmHg or map < 70mmHg for at least one hour despite adequate fluid resuscitation or adequate intravascular volume status, the need for vasopressors to maintain systolic blood pressure > 90mmHg or map > 70mmHg. The first organ or system dysfunction had to develop within 24hours before study enrolment. SOFA scores were performed daily through the 28day period.

Table 3 The SOFA score

SOFA score	1	2	3	4
Respiration				
PaO_2/FiO_2, mmHg	<400	<300	<200	<100
			—— with respiratory support ——	
Coagulation				
Platelets $\times 10^3/mm^3$	<150	<100	<50	<20
Liver				
Bilirubin, mg/dl	1.2–1.9	2.0–5.9	6.0–11.9	>12.0
(μmol/l)	(20–32)	(33–101)	(102–204)	(<204)
Cardiovascular				
Hypotension	MAP < 70 mmHg	Dopamine ≤5 or dobutamine (any dose)[a]	Dopamine >5 or epinephrine ≤0.1 or norepinephrine ≤0.1	Dopamine >15 or epinephrine >0.1 or norepinephrine >0.1
Central nervous system				
Glasgow Coma Score	13–14	10–12	6–9	<6
Renal				
Creatinine, mg/dl	1.2–1.9	2.0–3.4	3.5–4.9	>5.0
(μmol/l) or urine	(110–170)	(171–299)	(300–440)	(>440)
output			or <500 ml/day	or <200 ml/day

[a] Adrenergic agents administered for at least 1 h (doses given are in μg/kg·min)

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Table 1.1 The SOFA score (Vincent *et al.*,1996) [230]

Time to resolution of organ dysfunction was defined as the time patients with a baseline SOFA score higher than 0 took to reach a score of 0. This was then analysed over days 1-7 using Cox regressions with censoring at death or discharge. Of the 1690 intention to treat population, 1271 had MOD at study entry. In patients with MOD, the time to resolution of organ dysfunction over the first 7 days was significantly shorter in the treated group than in the placebo group for cardiovascular dysfunction p=0.006, hazard ratio 1.2. At 7 days 61.2% of treated patients with cardiovascular dysfunction at baseline (n=593) had resolved compared with 54.2% of placebo patients with cardiovascular dysfunction at baseline. Association with bleeding was more often during the infusion period.

Data relating to the development, course and severity of organ dysfunction were collected as secondary end points in the Prowess Trial [231]. Organ dysfunction was evaluated prospectively using modified SOFAs and the effect of APC on organ function and recovery studied to gain insight in to how the agent produced a mortality benefit. Reason for analysis of days1-7 included the 4 day infusion period and short half-life of APC making it likely that any observed effects would occur during or just after the infusion, less missing data as patients were on the ICU, less confounding morbidity and mortality. Sequential assessments of organ dysfunction during the first few days of ICU have been shown to be good indicators of prognosis [232]. Sepsis related organ dysfunction at baseline involving two or more organ systems was present in75% of the cohort, three or more in 43% and four or more in 18%. Cardiovascular dysfunction was present in the majority of patients and was commonly severe SOFA=3/4. Mean cardiovascular dysfunction scores averaged over days 1-7 and 1-28 were lower in the APC group compared with placebo (1.63vs 1.78 p=0.029 and 1.2 vs. 1.35 p=0.022). There were no significant differences for

respiratory, haematology or renal score. Evolution of organ dysfunction was analysed separately for patients with and without a particular organ dysfunction at baseline. For patients with organ dysfunction at baseline, APC treated patients had significantly greater likelihood of cardiovascular resolution (p=0.009) compared with placebo during days 1-7. Resolution of organ dysfunction on the first week was associated with improved 28 day survival. Analysis of aggregate morbidity showed that APC was associated with a significant reduction in cardiovascular dysfunction relative to placebo. Time to event analysis demonstrated more rapid improvement in cardiovascular function. Improved organ function was suggested to contribute to the observed mortality benefit of APC treatment as there was no increased morbidity for APC treated survivors compared with placebo survivors. Mechanisms for improved function remain to be investigated.

Cardiac troponin I (cTnI) and cardiac troponin T (cTnT) are highly sensitive and specific biomarkers for myocardial injury released as a result of myocardial cell injury [233, 234]. They are the regulatory proteins for cardiac muscle actin myofilaments and differ from isoforms found in skeletal muscle. Elevated levels have been found in patients with sepsis and shown to indicate left ventricular dysfunction with a poor prognosis [235].This was a retrospective study of patients with severe sepsis on the ITU in whom troponin levels had been measured. Inclusion criteria included measurement of troponin within the first 3 days of ICU admission. Cardiac failure was defined as a systolic blood pressure of lower than 90mmHg for at least one hour after adequate fluid resuscitation and/or the need for vasopressors to maintain a systolic blood pressure above 90mmHg. 105 patients had severe sepsis and troponin measured of whom 48 were cTnI positive. Patients who had troponin measured had higher APACHE II and MODS but the frequency of coronary artery

disease did not differ significantly. The primary end point of the study was ITU mortality which was 25/48 (52%) of the cTnI positive group compared with17/57 (30%) in the cTnI negative group (p=0.03). The 28 day mortality was also higher however ITU and hospital lengths of stay were similar. It was felt that the elevated troponins were not likely to be due to coronary artery disease alone as ECG changes were nonspecific and only 3 patients had wall motion abnormalities on ECHO. A similar frequency of ECG/ECHO abnormalities were observed in patients with normal troponin levels. High levels of tumour necrosis factor alpha and IL-6 have been shown in patients with elevated troponins suggesting a role in troponin release [236]. APC treatment of patients with an elevated troponin was associated with a reduction in mortality from 72-30%.

1.13 Thrombin and Protease Activated Receptors

Thrombin was named and identified as an agent within the circulation causing clotting, at the end of the nineteenth century. The protein was purified and sequenced in the 1950s and since then this serine protease has been recognized as a multifunctional serine protease central to haemostasis causing blood coagulation and regulating platelet aggregation[237]. In addition to coagulation, thrombin exerts a number of receptor mediated cellular effects as summarized in the diagram below;

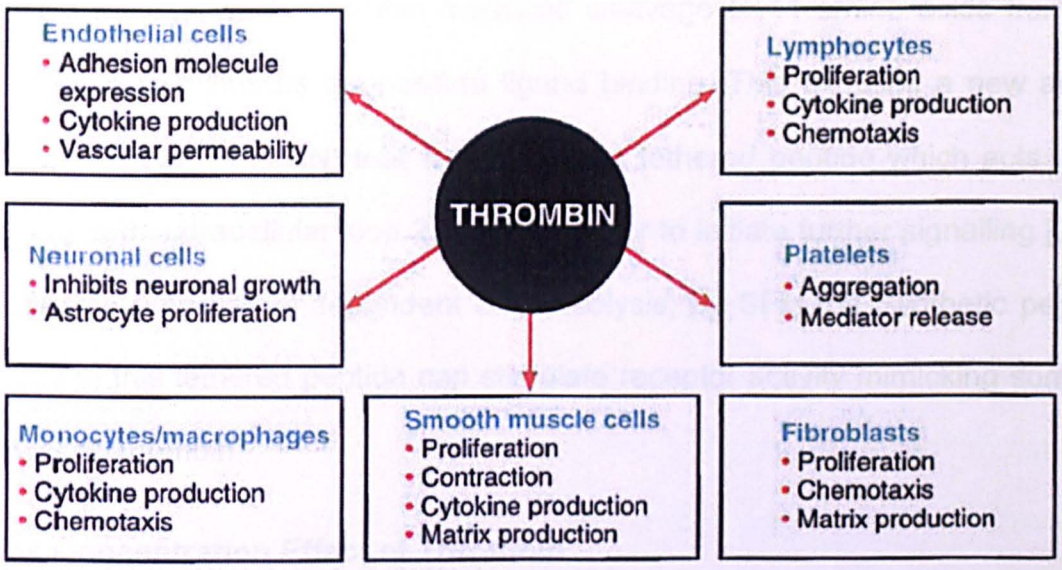


Figure 1.4 The variety of cellular effects stimulated by thrombin (Goldsack *et al,* 1998) [237] Used with permission from corresponding author

In rat ventricular myocytes, thrombin stimulates phosphoinositide hydrolysis, activates the extracellular signal–regulated protein kinase (ERK), induces atrial natriuretic factor expression, modulates calcium homeostasis, increases automaticity, and hastens recovery from an imposed acid load by activating Na^+-H^+ exchange [238-240]. Collectively, these (and likely other signalling events) profoundly alter electrophysiological properties and contractile behaviour and induce cardiomyocyte hypertrophy. Ultimately, the significance of these findings rides on the identification of natural activators of endogenous cardiomyocyte PARs.

Four PARs have been identified. PAR-3 is not present on human platelets but widely expressed in other cell types. In the mouse signalling in platelets is mediated entirely by PAR-4 with PAR-3 facilitating PAR-4 clevage at low thrombin concentrations. PAR-1 and PAR-3 are thrombin receptors and activation of PAR-4 requires PAR-3 as a thrombin binding cofactor [154, 241, 242]. PAR-1 is the most widely studied thrombin receptor, a member of the G-protein coupled receptor superfamily.

Activation occurs through thrombin mediated cleavage of 41 amino acids from the extracellular N-terminus as opposed to ligand binding. This exposes a new amino terminal sequence (SFLLRN) that functions as a tethered peptide which acts intra-molecularly with extracellular loop 2 of the receptor to initiate further signalling [243]. PAR-1 is also activated, independent of proteolysis, by SFLLRN Synthetic peptide analogues of this tethered peptide can stimulate receptor activity mimicking some of the effects of thrombin.

1.14 The Concentration Effect of Thrombin

In a regulatory role of inflammation, thrombin up-regulates the expression of mediators such as IL1, CAMs and PDGF in HUVECs. In primary endothelial cells, Feistritzer and Riewald showed protective activity at thrombin concentration 50pM in contrast to a proinflammatory role at 100pM using *in vitro* permeability assays [244]. In HPAECs Bae *et al.* demonstrate anti-inflammatory activities at 50pM (low concentration) including barrier protection, leucocyte adhesion inhibition and reduced expression of CAMs through activation of PAR-1 and PI3K pathways. Previous studies have shown barrier protective for APC mediated via the PI3K pathway. The barrier protective function of thrombin 50pM in HUVECs or 75pM in HPAECs was effectively suppressed by a cell permeable PI3K inhibitor [245].

1.15 APC VS. Thrombin and the Role of PAR-1

In 2003, Riewald *et al* showed that APC activated PAR-1 and 2 in an EPCR dependent manner [246]. PAR-1 deficient fibroblasts were responsive to 20nM APC only when EPCR was co-expressed with a PAR. Similar to thrombin, APC signalling was inhibited by PAR-1 clevage blocking antibodies whereas anti PAR-1 antibodies did not prevent signalling by the direct PAR-1 agonist or an unrelated GPCR agonist

excluding nonspecific PAR-1 desensitisation. In endothelial cells, APC induced MAPK phosphorylation via PAR-1 and 2. APC, but not thrombin responses, were inhibited by a 10 fold molar excess of active site blocked APC that competes for EPCR binding confirming receptor dependence of APC signalling in endothelial cells. Using high density micro-array analysis it was found that stimulation of HUVECs with APC or PAR-1/2 agonists induced most genes to a similar extent with the exception of MCP-1 induced only by the PAR-1 agonist. These results prompted the question of how can APC signalling by the prototypical thrombin receptor PAR-1 be relevant where the generation of APC is thrombin dependent.

Ruf reported how thrombin activates PAR-1 to elicit a multitude of proinflammatory effects [247] and suggested that local concentrations, vascular bed gradients of TM and EPCR and haemodynamic forces were likely determinants of the relative importance of thrombin vs. APC dependent signalling.

Profiles of APC vs. thrombin responses in cytokine stimulated endothelial cells showed qualitative and quantitative differences suggesting a more favourable protective phenotype. APC suppressed some genes up regulated by thrombin e.g. down regulation of thrombospondin and p53 [248].

Comparing the kinetics of PAR-1 clevage by APC vs. thrombin on endothelial cells, thrombin is a potent activator of PAR-1 on endothelial cells and its presence at the endothelial surface leads to the generation of APC [249]. To compare the ability of APC and thrombin to cleave PAR-1, a PAR-1 cleavage reporter was expressed in HUVECs where alkaline phosphatase is released from the substrate AP-PAR-1 when cleaved by thrombin or other proteases. Its activity in cell conditioned medium provides a measurable index of PAR-1 cleavge. Thrombin 10pm-30nm was used

relating to a circulating concentration of 1-2um. APC, 1-100nm was used where circulating levels are generally less than 0.2nm (27) Thrombin was found to be 10^3 to 10^4 fold more potent than APC where 100nm APC resulted in 20% maximal PAR-1 clevage and 50% maximal PAR-1 clevage occurred at 0.1nm thrombin. Using phosphoinositide hydrolysis as an end point of PAR-1 signalling, increases were readily detected at thrombin concentrations of 0.1nm and above and APC triggered responses approximately equivalent to those elicited by 0.1nm thrombin. To compare the potency of both proteases to trigger PAR-1 mediated responses, gene induction was studied. Responses were qualitatively similar but again APC was significantly less potent. The lack of potency again questioned whether PAR-1 activation by APC contributes to any effect beyond that of thrombin. In this study, the authors suggested that their *in vitro* experiments may overestimate any difference due to the absence of natural inhibitors and flow effects terminating thrombin activity.

In 2005 Riewald and Ruf, looked at APC vs. thrombin PAR-1 signalling in cytokine perturbed endothelial cells proposing that in view of APC's beneficial effects in sepsis that this may differ. Flow cytometry demonstrated that treatment of HUVECs with TNFα for 5 hours led to an increase in PAR-2 expression whereas PAR-1 levels remained unchanged. Large scale gene expression profiling was performed in TNF primed HUVECs stimulated with either thrombin, APC or a PAR-1 specific agonist peptide. Thrombin caused up/down regulation of a specific higher number of genes and this was related to its greater signalling strength. Thrombin was a more potent inducer of in particular tissue factor. Of note thrombin and APC selectively induced transcripts that were not induced by the other protease. A number of genes were suppressed by APC including pro-inflammatory and proapoptotic genes such as p53. APC also reduced thrombospondin protein levels in a PAR-1 cleavge dependent

manner in contrast to the inducing effect of thrombin suggesting that EPCR co-signalling may allow mediation of opposite biological effects of two proteases activating the same receptor. PAR-1 however is not universally colocalised to EPCR suggesting why even high concentrations of APC cannot desensitise subsequent thrombin responses. The authors concluded by reporting that APC and PAR agonist peptides have similar effects on gene expression in non-perturbed endothelial cells distinct from quite different properties under stimulated conditions and relate this to the therapeutic efficacy of APC in severe sepsis vs. earlier clinical stages.

Continuing the debate of how APC-PAR-1 signalling can be relevant where APC generation is thrombin dependent and thrombin is significantly more efficient as a PAR-1 activator, Feistritzer *et al* study explored whether the protective signalling by endogenously generated APC is enhanced when compared with exogenous APC. Having previously shown that very low concentrations of thrombin (40pm) can increase barrier integrity of a subconfluent layer of endothelial cells, it was tested whether endogenous APC could support barrier protection. An additive effect was found with a protective effect in response to 20pm thrombin found only in the presence of PC. The group also used HCAECs and showed that protective signalling in response to 2nm exogenous APC was comparably effective with the Eahy926 cell line. Using a thrombin variant with specificity favouring PC activation, they show that the endogenous PC activation pathway at the endothelial cell surface is linked to PAR-1 clevage dependent barrier protection by generated APC. They further suggest that the PC pathway may be the relevant activator of PAR-1 in response to low thrombin concentrations where thrombin in vivo activity is tightly controlled and thrombin inhibitors may shift responses from PAR towards PC pathway activation.

The work of Schuepbach *et al* [76] supports our findings that APC can mediate significant PAR-1 cleavage in the presence of thrombin. Using a model of endothelial barrier integrity, APC enhanced barrier function despite the presence of up to low nanomolar thrombin concentrations (1.56nm). Whereas thrombin cleaved PAR-1 is rapidly internalised and degraded, this group shows that APC activated PAR-1 remains on the cell surface and accumulates under prolonged incubation even when thrombin is present. In contrast thrombin cleaved PAR-1 is rapidly internalised and degraded [250].

Previous studies have shown that both native and cleaved PAR-1are translocated to the endothelial cell surface from intracellular pools following agonist treatment [251, 252]. This study reports that inefficient removal of APC cleaved PAR-1 from the cell surface and that APC cleaved PAR-1 may accumulate even in the presence of thrombin (up to1nm). The group went on to demonstrate that APC cleaved PAR-1 was surface retained in cytokine perturbed cells pre-treated with high dose TNF (5nM). Proposed reasons for the differences in downstream signalling pathways following G protein coupling of PAR-1 for different agonists include; cleavage rate, surface retention of PAR-1 and distinct signalling for EPCR colocalised PAR-1.

1.16 Summary

APC is an endogenous anticoagulant with anti-inflammatory properties. Therapeutic administration of APC had emerged as the first treatment for patients with severe sepsis. With concerns over its haemorrhagic risk, improved understanding into its anti-inflammatory and cytoprotective mechanisms holds promise for further improvement in outcome for patients that may extend beyond the clinical confines of severe sepsis. As described above, improvement in cardiovascular parameters

during APC treatment patients in severe sepsis are highly relevant to overall patient outcome. However, much remains unclear about the role of APC on cardiac cells both at the coronary endothelial and contractile level.

The overall hypothesis of this thesis is that APC can have a positive effect and improve cardiac function directly at the cellular level. The specific hypotheses are:

1. APC can induce anti-inflammatory and cytoprotective properties on human coronary artery endothelial cells.

2. APC can induce an EPCR and PAR-1-dependent positive inotropic effect on the heart via direct action on cardiomyocytes.

The experimental plans to address these specific hypotheses are detailed within the chapters of this thesis.

Chapter 2 Materials and Methods

Human Coronary Artery Endothelial Cells (HCAECs)

2.1 Materials

HCAEC C-22020, DetachKit, HCAEC media plus supplementix – PromoCell, Sickingenstr. 63/65 69126 Heidelberg Germany, RNeasy Mini Kit and QIA Shredder – Qiagen, Activated Protein C, 1st strand cDNA synthesis kit for RT-PCR (AMV) – Roche Cat no 11483188001, SuperArray Bioscience Corporation – Tebu-bio, USA, LightCycler FastStart DNA Master SYBR Green I – Roche

2.2 Methods

2.2.1 Maintenance and subculture of HCAECs

A T25 cell culture flask was preheated with 5ml of media in an incubator (37°C, 5% CO_2) for 30 minutes. Immediately after arrival, the cryovial of cryopreserved cells were thawed in a water bath (37°C) and then the cryovial was rinsed with 70% ethanol to avoid microbial contamination. After wiping the vial with a tissue, it was opened under a laminar flow bench and the cells resuspended by carefully pipetting up and down. The cells were transferred to the T25 containing pre-warmed medium and then placed in the incubator (37°C, 5% CO_2) for cell attachment. The medium was replaced after 16–24 hours. The cells were then subcultured once they had reached 70–90% confluency. The PromoCell DetachKit was placed at room temperature for at least 30 minutes to adjust the temperature of the reagents. The medium from the T25 containing the proliferating cells was then carefully aspirated. 3ml Hepes BSS Solution was added to wash the cells and then the vessel agitated carefully for 15 seconds. The Hepes BSS was then carefully aspirated from the T25.

3ml Trypsin/ EDTA Solution was added and then the closed vessel of cells examined under a microscope. Following the start of cell detachment, the side of the vessel was gently tapped to loosen the remaining cells. 3ml Trypsin Neutralization Solution was added followed by gentle agitation. The cell suspension was carefully aspirated and transferred to a centrifugation tube. The cells were spun down for 3 minutes at 220 x g, the supernatant discarded, 1 ml of the appropriate PromoCell Cell Growth Medium added (step 2) and the cells resuspended by carefully pipetting up and down. The cells were plated according to the recommended seeding density in new cell culture vessels containing PromoCell Cell Growth Medium prewarmed to 37°C and in an incubator (37°C, 5% CO_2).

2.2.2 RNA Isolation and Concentration Determination

The monolayer of cells was directly lysed using 700µl RLT buffer (Qiagen) and homogenised by vortexing for 1 min. The lysate was pipetted the into a QIAshredder spin column placed in a 2ml collection tube and centrifuged for 2 minutes at full speed in a microcentrifuge. 1 volume of 70% ethanol was added to the homogenized lysate, and mixed well by pipetting up and down. 700µl of the sample was transferred, including any precipitate that may have formed, to an RNeasy spin column placed in a 2ml collection tube. The tube was centrifuge for 15 secs at ≥ 8000 x g (≥ 10,000 rpm), the flow-through discarded and 700 µl Buffer RW1 added to the RNeasy spin column. Following centrifugation for 15 s at ≥ 8000 x g (≥ 10,000 rpm) to wash the spin column membrane, the flow-through was discarded. The column was transferred to a new 2ml collection tube and 500µl Buffer RPE (supplemented with ethanol) added to the RNeasy spin column. Following centrifugation for 15s at ≥ 8000g (≥ 10,000 rpm) to wash the spin column membrane, the flow-through was discarded. 500µl Buffer RPE was added to the RNeasy spin

column and centrifuged for 2 min at ≥ 8000g (≥ 10,000 rpm) to wash the spin column membrane. The RNeasy spin column was then carefully removed from the collection tube so that the column did not contact the flow-through to prevent carryover of ethanol. The RNeasy spin column was in a new 2 ml collection tube (supplied), and the old collection tube discarded with the flow-through. Centrifugation was performed at full speed for 1 min to eliminate any possible carryover of Buffer RPE, or residual flow-through on the outside of the RNeasy spin column. The RNeasy spin column was then placed in a 1.5ml eppendorph (RNAse/DNAse-free), 30–50 µl RNase-free water added directly to the spin column membrane and centrifuged for 1 min at ≥ 8000g (≥ 10,000 rpm) to elute the RNA.

The concentration of the RNA yield was determined by mixing 2uL of RNA with 98uL $_dH_2O$ (1in 50 dilution) in a well of a 96 well UV plate and the optical density (OD) measured at 260nm and 280nm, together with 2 blank wells of 100uL $_dH_2O$ to zero the spectrophotometer. The RNA concentration and purity were calculated as below:

RNA concentration = OD_{260} x 40 x 50 (dilution factor)

RNA purity = OD_{260}/ OD_{280}

A concentration of 0.5ug/uL RNA and purity 1.8 is recommended for RT-PCR.

Volume of RNA required for RT-PCR = 500/ RNA concentration

2.2.3 cDNA Synthesis for RT-PCR

A master mix for the reaction was prepared as described below using the kit reagents.

Reagent	Volume/reaction
10 X Reaction Buffer	2uL
25mM $MgCl_2$	4uL
Gelatin	0.5uL
Deoxynucleotide mix	2uL
Oligo $p(dT)_{15}$ Primer	2uL
RNase Inhibitor	1uL
AMV Reverse Transcriptase	0.8uL

On ice, 12.3uL of master mix per sample was mixed with the RNA (as calculated as above) in a total reaction volume of 20uL, using $_dH_2O$ to make up the remaining volume. The microcentrifuge tube was briefly vortexed and centrifuged and then the reaction incubated at 25°C for 10 minutes, 42°C for 60 minutes, 99°C for 5 minutes, and 4°C for 5 minutes. DNA samples were then stored at -20°C for future use.

2.2.4 Polymerase Chain Reaction (PCR)

Semi-quantitative PCR

Each specifically amplified cDNA sample was diluted 1 in 10 with loading buffer and loaded on to a 1% agarose gel (plus ethidium bromide) including a relevant housekeeping gene e.g. GAPDH, marker and controls. The gel was run at 120V for 45 minutes and then visualised under UV light.

Quantitative RT PCR

LightCycler Carousel-Based System Protocol:

The reaction mixture was prepared on ice as below, and then gently mixed:

Component	**Volume/ 15uL reaction**
Water, PCR-grade	9uL
Forward Primer (0.2-1uM)	1uL
Reverse Primer (0.2-1uM)	1uL
SYBR green	4uL

(A negative control was always run replacing the template DNA with PCR-grade water.)

15uL of the reaction mixture was pipetted into a LightCycler capillary and 5uL of the DNA template added. Each capillary with sealed with a stopper and then briefly centrifuged before being transferred to the LightCycler Sample Carousel. The Carousel was placed in the LightCycler 2.0 Instrument and the programmed protocol started. PCR was confirmed by only the desired product being amplified using a

melting curve (only one peak should be apparent with primer dimer or non-specific products presenting as additional melting peaks) and using gel electrophoresis.

2.2.5 Fluorescence Activated Cell Sorting (FACS) for Expression of EPCR

The medium from one T25 flask of confluent cells was removed, the cells washed once with PBS, 2ml versene added and the flask incubated at 37°C for 5 minutes. The cells were detached by tapping the flask and transfer to a universal. The flask was washed with 3ml media to remove any remaining cells and this was then combined with the cells in the universal. The universal was spun at 500g for 4 minutes at room temperature, the supernatant discarded and the cells resuspended in 1ml PBS. The cells were then transferred to a 1.5ml eppendorf and spun at < 1000g for 30 seconds. The supernatant was removed and the cells resuspended in 600uL PBaz. 100uL cells were aliquoted in to 6 separate 1.5ml eppendorfs to perform each specific stain in triplicate i.e. use 3 tubes for class control and 3 for EPCR. The tubes were spun in the minifuge at <1000g for 30secs and then the supernatants removed. The cells were resuspended in 50uL of appropriate primary antibody diluted 1:200 in PBaz (IgG2a or EPCR). After incubating the cells on ice for 20 minutes, the cells were spun at <1000g for 30secs, the supernatants removed and cells washed twice in 100uL PBaz. The cells in were resuspended in 50uL of the secondary antibody (Anti-rat IgG-FITC), diluted 1:50 in PBaz, and incubated on ice for a further 20 minutes, in the dark. The cells were then spun down at <1000g for 30secs and washed once in 100uL PBaz. Finally, the cells were resuspended in 100uL of FACS flow and transferred to FACS tubes containing 300uL FACS flow for FACS analysis.

2.2.6 Isolation of MPs from APC Conditioned Media

The cells were induced overnight with 100nM APC in 0.2um filtered 2%FCS media, collected, and spun at 180g for 10 minutes at 4°C to pellet cell debris. Supernatant was transfer to a fresh tube and spun at 18,000g for 15 minutes at 4°C. The supernatant was removed leaving about 20uL at the bottom of the tube so as not to disturb the MP pellet. The MPs (MPs) were resuspended in 500uL PBS (0.2um filtered) and spun at 18,000g for 15 minutes at 4°C. Again supernatant was removed and then the pellet resuspended in 100uL filtered PBS. MPs could be stored for a maximum of 2 days at 4°C but were used straight after isolation

2.2.7 Estimation of MP Bound APC concentration

A standard curve was constructed using APC concentrations of 200, 100, 50, 25, 12.5, 6.25, 3.125 and 0nM in PBS. 50uL duplicate standards and samples (1 in 5 dilution) were prepared in a micro-titre plate and combine with 50ul of chromogenic substrate S2366. A kinetic spectrophotometric analysis was performed at 405nm every 5 minutes for 1 hour. The MP APC concentrations were calculated from the standard curve using the plate reader software provided (Gen5).

2.2.8 Stimulation of HCAECs with TNF

Confluent cells of equal passage were used per experiment. After washing with PBS, the HCAECs were stimulated with TNF (diluted in HCAEC medium) at t = 0 +/- APC at t = 4hrs. The cells were incubated until t = 24hrs (37°C, 5% CO2) and then harvested.

2.2.9 Microarray Probe synthesis

For each RNA sample, the annealing mixture was prepared as described below in a sterile PCR tube:

Component	Volume/ 10uL reaction
Total RNA	0.5ug
Buffer P	1uL
RNase-free H_2O	Adjust final volume to 10uL

The mixture was vortexed and briefly centrifuged before placing in a thermal cycler at 70°C for 3mins and held at that temperature for 10mins.

The RT cocktail was prepared as described below then heated at 37°C for 1 min before proceeding.

Component	Volume/array
Buffer BN	4ul
RNase-free H_2O	4ul
RNase Inhibitor	1ul
Reverse Transcriptase	1ul

For each array, 10uL of the RT cocktail was transferred to the 10uL annealing mixture, mixed well and incubation continued at 37°C for a further 25mins. Heating then occurred at 85°C for 5mins to hydrolyse the RNA and inactivate the reverse transcriptase. The finished RT reaction was held the on ice until the next step.

The LPR Cocktail was prepare as described below:

Component	**Volume/array**
Buffer L	18uL
Buffer AF	9uL
Biotin-16-dUTP	2uL
DNA Polymerase	1uL

For each array, 30uL of the LPR cocktail was added to each RT reaction and mixed well.

The thermal cycler was programmed as follows; 85°C for 5mins, 30 cycles of (85°C for 1 min, 50°C for 1 min, 72°C for 1min) and then 72°C for 5mins.

The labelled cDNA probe was denatured and the reaction stopped by heating the tubes containing the LPR at 94°C for 2mins and then quickly chilling on ice. Probes could be stored temporarily at -20°C before proceeding with hybridisation.

2.2.10 Hybridisation

The membrane was pre-wet by adding 5 ml $_{d}$H2O to the hybridisation tube for 5 min. The tube allowed to sit inverted whilst preparing the GEAprehyb. The GEAprehyb solution was warmed to 60°C and the bottle inverted several times to dissolute the buffer components. The Salmon Sperm DNA was heated at 100°C for 5 min, then immediately chilled on ice and added to GEA Hybridization Solution at 100 mg/ml.

$_{d}$H2O was discarded from the hybridisation tube and 2 ml of the GEA Hybridization .Solution added before vortexing the tube for a few seconds. The tube was then

placed inside the hybridisation cylinder and pre-hybridized at 60°C for 1-2 h with continuous agitation at 5-10 rpm.

The entire volume of denatured cDNA probe for the AmpoLabeling-LPR was added to 0.75ml of pre-warmed GEAprehyb. This was mixed and kept at 60°C. The GEA Hybridization Solution was discarded from the hybridisation tube and added to the GEAprehyb (containing probe). This was incubated overnight at 60°C with continuous agitation at 5-10rpm.

2.2.11 Washing and Chemiluscent Detection

Wash solutions 1 and 2 were prepared and warmed to 60°C. 10ml was used per hybridisation tube. The membrane was washed twice with 2X SSC, 1% SDS for 15 min with 20-30rpm agitation at 60°C. The membrane was then washed a further twice with 0.1X SSC, 0.5% SDS for 15 min with 20-30rpm agitation at 60°C.

After discarding the last wash, the membrane was blocked for 40 min with 2 ml of GEAblocking solution Q with continuous agitation at 20-30rpm. 1X Buffer F (18 ml per array) was prepared by diluting 5X with dH2O. 1:8,000 AP-SA was prepared by dilution (2 ml per array) in 1X Buffer F. The array was incubated with 2 ml of AP-SA dilution for 10 min then the membrane washed 4 times with 4 ml 1X Buffer F 5 min each. It was then rinsed twice with 3 ml Buffer G and the membrane incubated at room temperature in the hybridisation tube with 1.0 ml CDP-Star for 2 to 5 min. The membrane was blotted on a piece of filter paper to remove any excess CDP-Star solution. The membrane should not be allowed to dry out. After insertion into a plastic sheet protector and smoothing of any bubbles image acquisition was performed using a cooled CCD camera. The web-based integrated GEArray Expression Analysis Suite was used for data analysis.

2.2.12 APC Antibody blocking Experiments

Confluent cells of equal passage were used per experiment. After washing with PBS, the HCAECs were stimulated with TNF or LPS (diluted in HCAEC medium) at t = 0 +/- APC at t = 4hrs. The cells were incubated until t = 24hrs (37°C, 5% CO2) and then harvested.

2.2.13 Statistics

All data were statistically analysed from at least 3 independent experiments unless otherwise specified. Two group comparison before and after treatments used students t test.

Rat Ventricular Cardiomyocytes

2.3 Materials

Solution A (in mmol/L): NaCl 130, KCl 5.4, $MgCl_2$-$6H_2O$ 1.4 (1.4 mL of 1M $MgCl_2$ solution for 1 litre of solution A), NaH_2PO_4 0.4, Creatine 10, Taurine 20, Glucose 10, HEPES 10, pH to 7.3 with NaOH.

Extracellular control solution (ECS) (in mmol/L): NaCl 140, KCl 5.4, $CaCl_2$ 1, $MgCl_2$ 1, D-glucose 10, HEPES 10, adjusted to pH 7.35.

HEPES-Tyrode buffer referred to as low Ca^{2+} Solution: 500 ml of Solution A + 375 µL of 1M $CaCl_2$ stock solution.

EGTA solution: 250 ml of solution A + 250 µL of 0.1M EGTA stock solution

Enzymatic solution: 50 ml of solution A + 25 µL of 0.1M $CaCl_2$ stock solution, 35mg of collagenase type 1 and 1.5mg of protease type XIV

BSA Solution: 5 ml of solution A + 2.5µL of 0.1M $CaCl_2$ stock solution + 0.5g of BSA

All chemicals were purchased from Sigma except collagenase type I purchased from Worthington and $CaCl_2$, $MgCl_2$ stock solutions purchased from BDH Laboratory Supplies.

Antibodies: anti-PAR-1 (ab32611) from Abcam (Cambridge, UK), anti-PAR-1 (WEDE15) from Beckman Coulter, anti-PAR-1 (ATAP2), anti-EPCR (P20), goat anti-rabbit IgG-HRP and donkey anti-goat IgG-HRP from Santa Cruz Biotechnology (CA, USA) and RCR252 (EPCR blocking antibody) from Dr Kenji Fukudome (Saga Medical School, Japan). The PAR-1 blocking peptide H-2514 was from Bachem (St Helens, UK). Immunohistochemistry utilised anti mouse Extravidin-Peroxidase staining kit (Sigma) and DAB liquid (DAKO, USA). ECL Advanced Blocking Reagent kit was purchased from GE Healthcare (Little Chalfont, UK).

Human lyophilised platelets were purchased from Bio/Data Corporation (Horsham, USA), human APC from Haematologic Technologies Inc (Vermont, CA), human alpha thrombin Haematologic Technologies Inc., ethyleneglycol-bis (β-aminoethyl)-N,N,N',N'-tetra acetic acid (EGTA), type XIV Protease and calcium ionophore 4-bromo-A-23187 from Sigma (Gillingham, UK), collagenase type I from Worthington Biochemical Corporation (Lakewood, NJ 08701, USA), monofilament nylon cloth from Caddish (UK) and acetoxymethyl-ester-fura-2 (Fura-2-AM) from Invitrogen Molecular Probes (Eugene, Ore, USA)

2.4 Methods

2.4.1 Preparation of the apparatus

Left ventricular myocytes were obtained from male Wistar rats (200-300g) according to methods described previously [253]. Isolations were performed wearing protective gloves, coats and a mask according to animal handling regulations. The isolation apparatus is shown below in Figure 2.1.

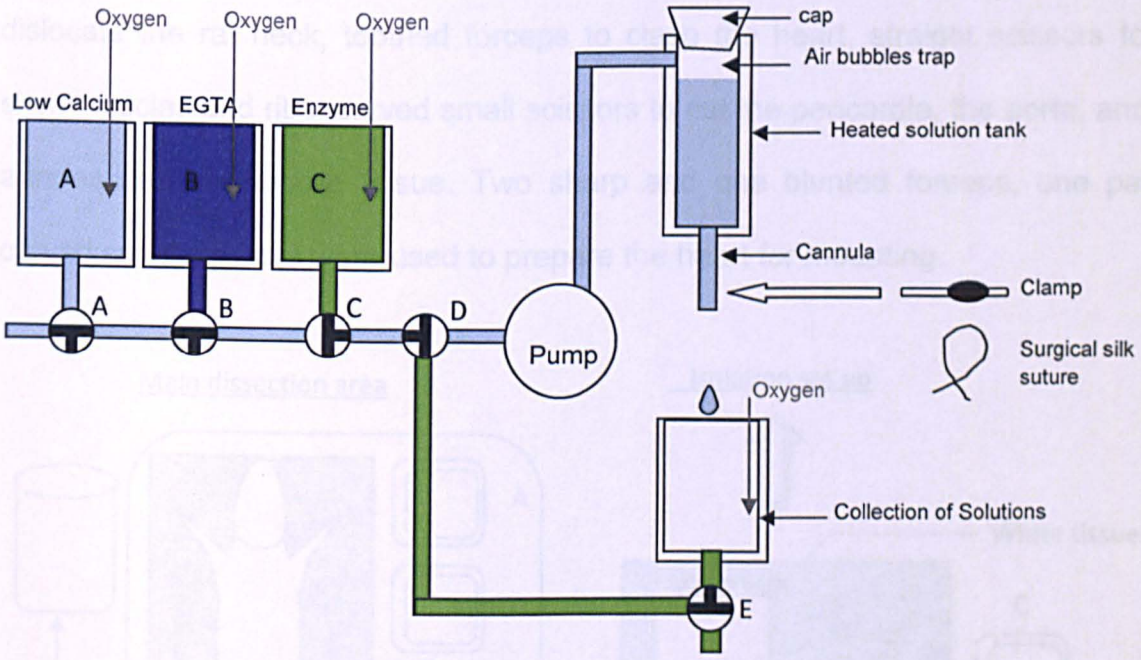


Figure 2.1 Cardiomyocyte isolation apparatus A, B and C represent oxygenated organ chambers filled with the solutions given above. Following excision from the rat, the heart is mounted on the cannula and perfused sequentially by the solutions linked to the tank.

The apparatus was washed prior to use by perfusing with distilled water. The organ chambers were oxygenated and preheated from an adjacent water bath set to 37°C.

The organ chambers A, B and C were consecutively filled with low calcium solution, EGTA and enzymatic solution respectively. Subsequently the tubing was pre-filled as shown in Figure 2.1. The clamp and surgical suture were prepared at this stage prior to cannulation of the heart.

2.4.2 Preparation for dissection of the heart

Having set up the dissection area as shown in Figure 2.2, the weighing boats A, B and C and a beaker were filled with cold low calcium solution. The instruments used were employed for the following: long surgical scissors blunted and curved used to dislocate the rat neck, toothed forceps to clasp the heart, straight scissors to cut skin, muscles and ribs, curved small scissors to cut the pericardia, the aorta, and the arteries and connective tissue. Two sharp and one blunted forceps, one pair of curved small scissors were used to prepare the heart for mounting.

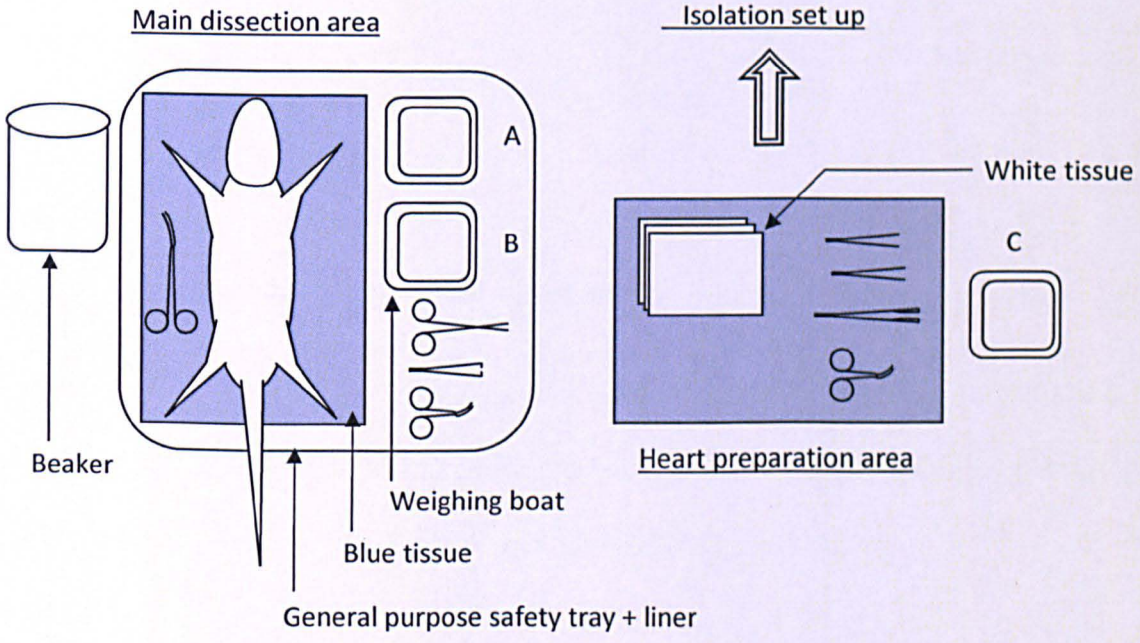


Figure 2.2 Layouts for excision and mounting of heart tissue

2.4.3 Excision and mounting of the heart

The rat was sacrificed following a schedule one procedure. This involved stunning the rat with a blow to the head followed by rapid dislocation of the neck. The thorax was opened as shown in Figure 2.3 cutting the skin and muscle layers below the diaphragm (a) and alongside the ribs to below the clavicle (b). The chest wall was pulled up to the thymus gland to expose the arteries and veins connected to the heart (c). The heart was then freed from the pericardium ensuring that the aorta had not been damaged during the dissection and was of a good length. The heart was transferred to weighing boat C before detachment from the oesophagus and lungs. This step should ideally be performed in less than one minute.

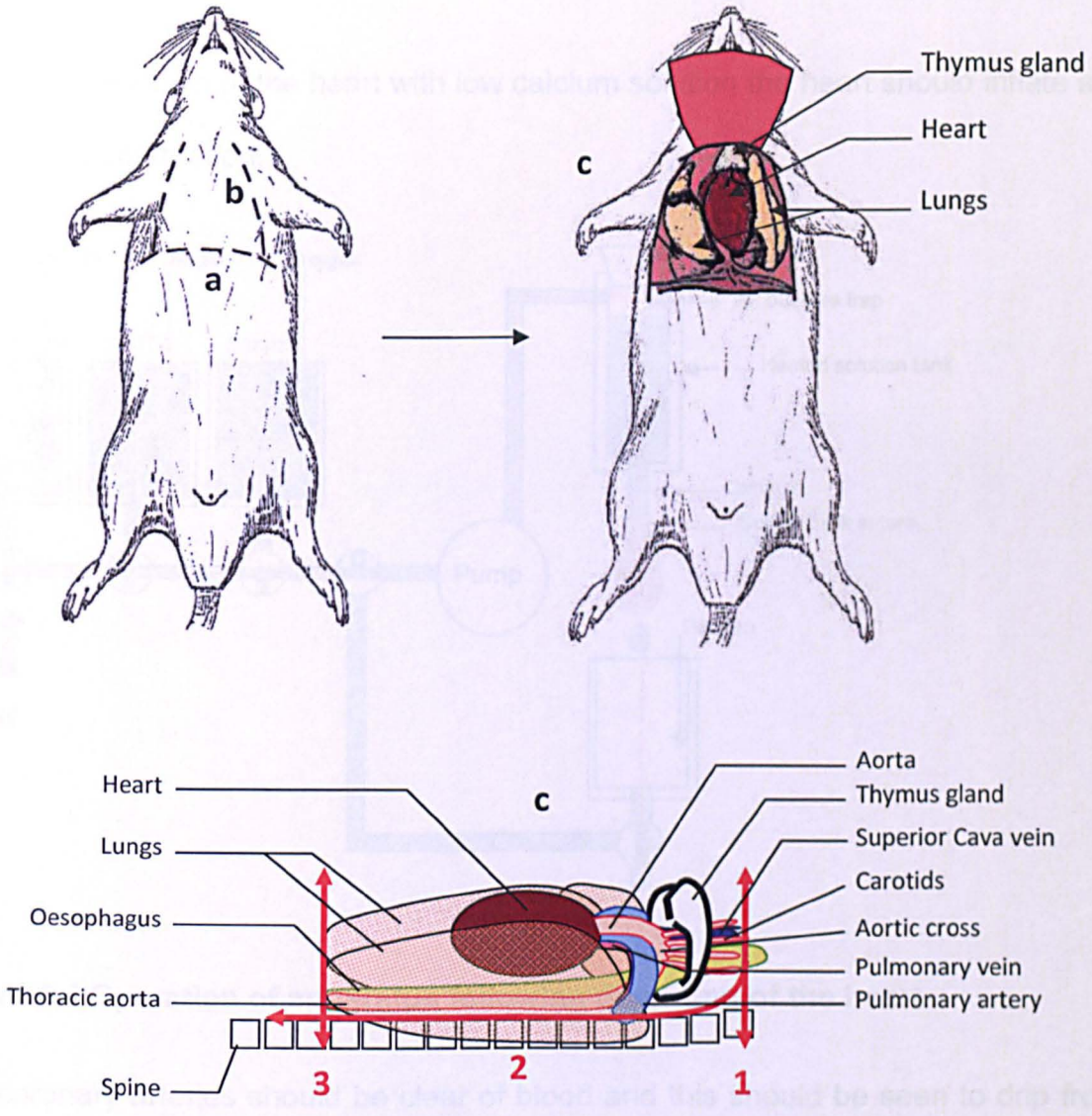


Figure 2.3 Schematic diagram of rat anatomy

The heart was mounted by sliding the aorta over the cannula (see Figure 2.4) carefully to avoid damage to the aortic valve. The heart was secured by clamping the aorta to the cannula and reinforced with the surgical silk suture.

2.4.4 Enzymatic Isolation of CMs

Following perfusion of the heart with low calcium solution the heart should inflate and commence contraction.

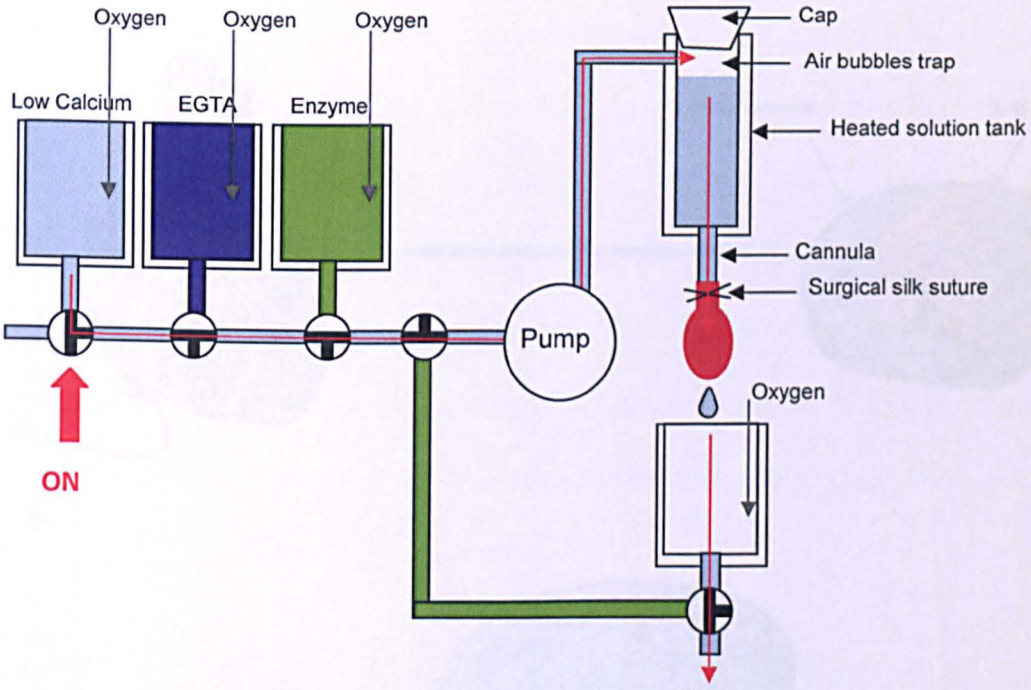


Figure 2.4 Operation of apparatus following mounting of the heart

The coronary arteries should be clear of blood and this should be seen to drip from the apex of the heart. After the solution has become clear (2-6 minutes) perfusion with the EGTA solution was continued for 4 minutes followed by enzymatic solution for 6 minutes. The heart should cease to contract 1- 2 minutes after starting perfusion with EGTA solution. The solution dripping from the heart can be observed to become oily due to the action of the collagenase.

The heart was removed from the perfusion system using the blunted forceps. By grasping the base of the aorta, the tissue distal to the tip of the cannula was cut to free the heart, which was then transferred to a weighing boat containing 1mL

enzyme solution. Connective tissue, arterial and venous tissue, and the atriums were removed as shown in Figure 5.

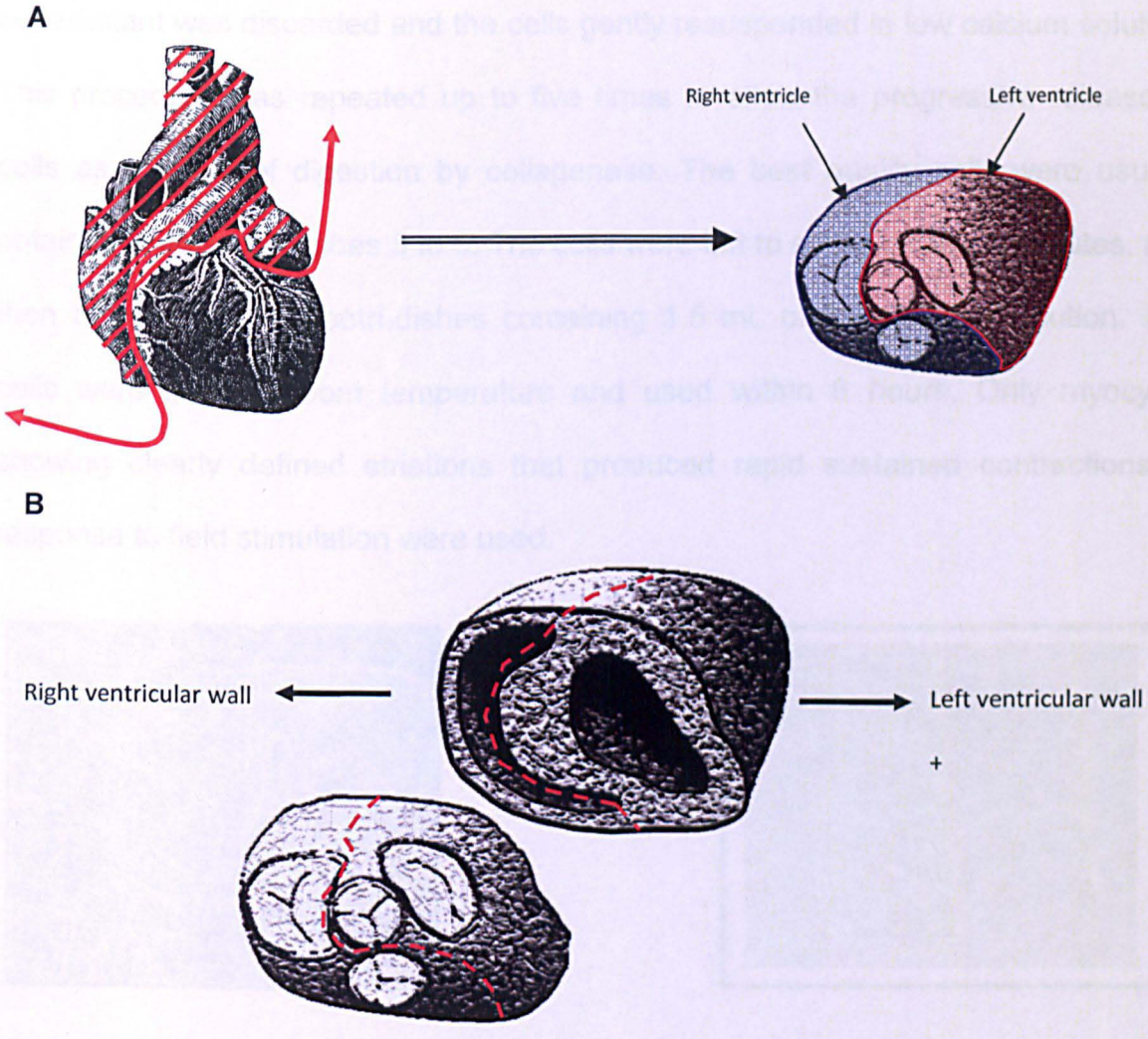


Figure 2.5 Heart dissection borders

The left ventricle and septum were excised (Figure 2.5) and then chopped 20 to 25 times in to small chunks of 1mm in size using the curved scissors.

35ml of recollected or freshly prepared enzyme solution was mixed with 5ml BSA aliquot and then 5ml of this solution added to the dissected chunks in a conical flask.

Following oxygenation and agitation (300-350 osc/min) for 4 minutes in the water bath, the flask contents were emptied through a funnel holding a folded nylon net square in to a collection test tube. Low calcium solution was added to a final volume of 10ml in the collection tube before centrifugation for 90 seconds at 500 rpm. The supernatant was discarded and the cells gently resuspended in low calcium solution. This procedure was repeated up to five times to allow the progressive release of cells as a result of digestion by collagenase. The best quality cells were usually obtained in collection tubes 3 to 5. The cells were left to settle for 10-15 minutes, and then transferred in to petri-dishes containing 1.5 mL of low calcium solution. The cells were kept at room temperature and used within 6 hours. Only myocytes showing clearly defined striations that produced rapid sustained contractions in response to field stimulation were used.

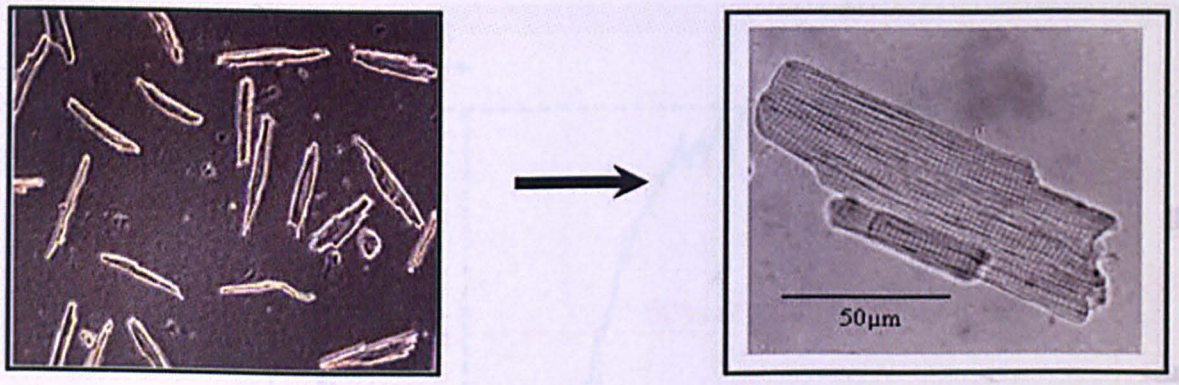


Figure 2.6 Cardiomyocytes; immediately following isolation, in solution (left) and single cardiomyocytes (right) as observed using video edge recognition system

2.4.5 Measurement of cardiomyocyte shortening

The effect APC and/or thrombin on unloaded contractions of ventricular cardiomyocytes was studied using a video edge recognition system (IonOptix, MyoCam-S, Dublin, Ireland) as previously described [254, 255]. Cardiomyocytes

were randomly selected and contractions recorded for a minimum of 1 min in the control state followed by addition of the test agent. Following the application of APC, continuous perfusion was maintained until a plateau in cardiomyocyte response was achieved. Following this, APC was discontinued and the cells perfused with Tyrode buffer solution until a return towards control state was observed. Experimentally the term 'shortening' represents cardiomyocyte contraction and has been used throughout the work. 10 consecutive shortenings were analysed for each test concentration and control state for each cell. The effect of perfusing thrombin (0.1 and 1U/mL), before, or after APC (5nmol/L) on the shortening response was also studied. Parameters derived included: amplitude of cell shortening, time to peak of shortening (TTP), maximum speed achieved during development of shortening (dS/dt) and time constant of relaxation (T).

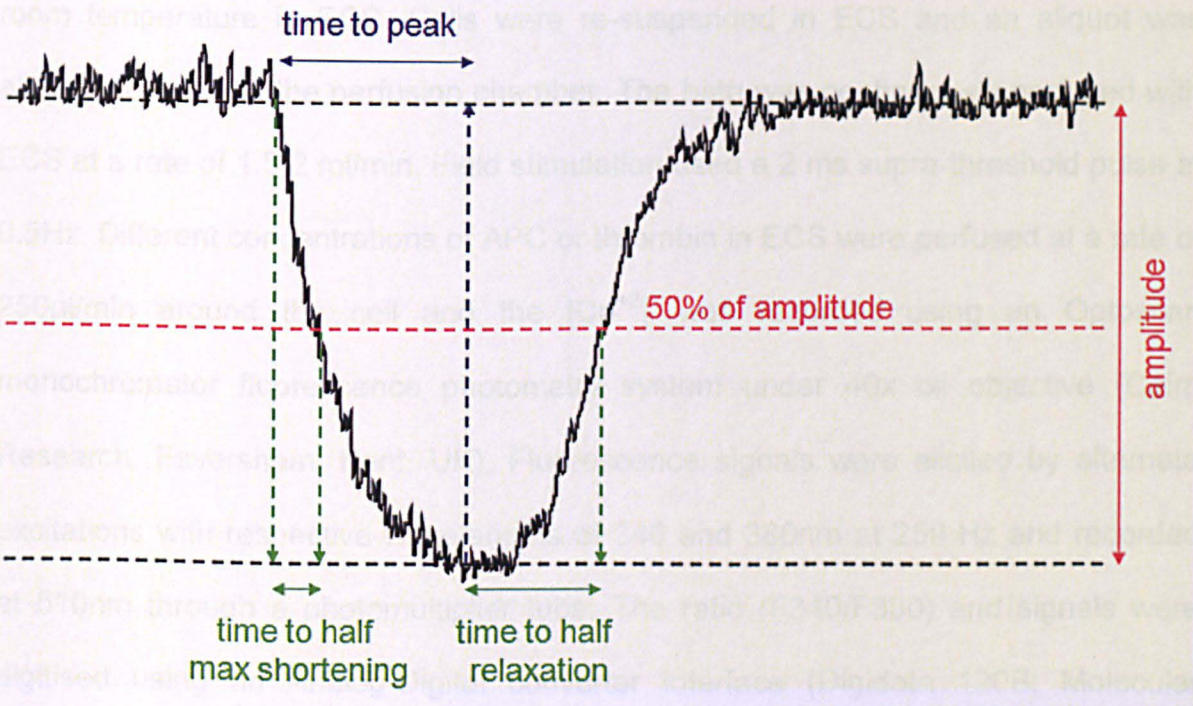


Figure 2.7 Cardiomyocyte shortening trace parameters; shortening amplitude (dS), maximum velocity (dS/dT) shortening gradient and time to peak (TTP) main

parameters used. (time to half max shortening and time to half relaxation also shown).

The induced responses were also measured in the presence of PAR-1 blocking peptide (H2514/ATAP2), PAR-1 antibody (WEDE15) or EPCR blocking antibody (RCR252). Cardiomyocytes were pre-incubated for between30 minutes and 1 hour with the blocking agent prior to delivery of APC. Zymogen protein C, glycerol and PBS (both APC vehicle buffers) were also tested separately as controls.

No serum was present during experiments and therefore it was assumed that thrombin was absent unless added exogenously.

2.4.6 Measurement of intracellular calcium $[Ca^{2+}]_i$

Following isolation, myocytes were loaded with Fura-2-AM (5μmol/L) for 15 min at room temperature in ECS. Cells were re-suspended in ECS and an aliquot was allowed to settle in the perfusion chamber. The bath was continuously perfused with ECS at a rate of 1.5-2 ml/min. Field stimulation used a 2 ms supra-threshold pulse at 0.5Hz. Different concentrations of APC or thrombin in ECS were perfused at a rate of 250μl/min around the cell and the $[Ca^{+2}]_i$ was recorded using an Optoscan monochromator fluorescence photometry system under 40x oil objective (Cairn Research, Faversham, Kent, UK). Fluorescence signals were elicited by alternate excitations with respective wavelengths of 340 and 380nm at 250 Hz and recorded at 510nm through a photomultiplier tube. The ratio (F340/F380) and signals were digitised using an Analog-Digital converter interface (Digidata 120B; Molecular Devices, US), sampled at 5 kHz and stored on a computer using Clampex 8.2 software (PClamp 8.2, Molecular Devices Corporation, US).

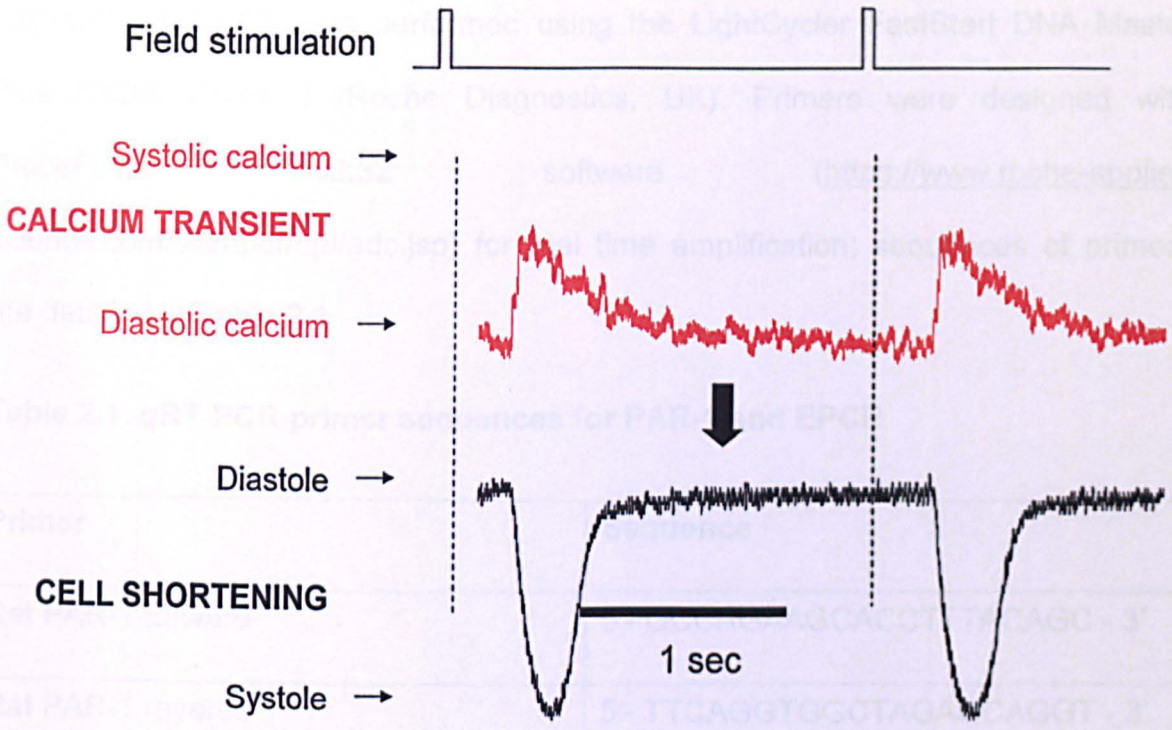


Figure 2.8 Schematic diagram of cardiac cycle for cardiomyocyte contraction; Following field stimulation of isolated cardiomyocytes, there is a rise in systolic Ca^{2+} as shown by the trace in red followed shortly afterwards by contraction (shortening) trace shown in black.

2.4.7 CM RNA preparation and PCR

All instruments were treated overnight with DepC H_20 and autoclaved. RNA was extracted from freshly isolated rat left ventricle. The tissue was chopped up finely, transferred straight in to RNA later solution followed by homogenisation in Trizol (Invitrogen). For optimal yield further steps of extraction using chloroform and isopropyl alcohol were performed followed by washing of the RNA pellet with 75% ethanol and then air drying. RNA integrity was determined by presence of the 18S and 28S ribosomal RNA species after electrophoresis. For semi-quantitative PCR, total RNA (500ng) was reverse-transcribed using First Strand cDNA Synthesis Kit (Roche) and used for specific amplification of PAR-1 and EPCR. For quantification

purposes, qRT-PCR was performed using the LightCycler FastStart DNA Master Plus SYBR Green I (Roche Diagnostics, UK). Primers were designed with ProbeFinder v2.32 software (https://www.roche-applied science.com/sis/rtpcr/upl/adc.jsp) for real time amplification; sequences of primers are detailed in Table 2.1

Table 2.1. qRT-PCR primer sequences for PAR-1 and EPCR

Primer	Sequence
Rat PAR-1 forward	5'- GCCAGAAGCACCTTTACAGC - 3'
Rat PAR-1 reverse	5'- TTCAGGTGGCTAGAGCAGGT - 3'
Rat EPCR forward	5'-GCCTCTGGGAAAACTCCT -3'
Rat EPCR reverse	5'- TCGCGATAGATCAACTGCAC – 3'
Human PAR-1 forward	5'-TACGCCTCTATCTTGCTCATGA-3'
Human PAR-1 reverse	5'-TTTGTGGGTCCGAAGCAAT-3'
Human EPCR forward	5'-GTAGCCAAGACGCCTCAGAT-3'
Human EPCR reverse	5'-GATAGGGGTCGCGGAAGTA

EPCR and PAR-1 expression was demonstrated by performing absolute quantification on the LightCycler (Roche Applied Science) using external standards of human and rat reference total RNA (0.16-100ng) (Stratagene). The amplification conditions were as follows: 45 cycles of 95°C for 15 seconds, 65°C for 10 seconds and 72°C for 12 seconds, followed by a melting cycle at 65°C for 15 minutes and cooling cycle at 40°C for 30 minutes, with acquisition of fluorescence at the end of

each extension. For each sample and gene, a threshold cycle (C_T) value was recorded. Standard curve generation and data analysis was performed using the LightCycler software provided.

2.4.8 Western Blot Analysis

Protein lysates were made from freshly isolated cardiomyocytes in SDS buffer (1% SDS, 10% glycerol, 50mmol/L Tris, 5mmol/L EDTA). Lysates were also prepared from human platelets (PAR-1 positive control), KOLF cells (PAR-1 negative control), EAhy926 cells (EPCR positive control) and LNCaP cells (EPCR negative control). Bio-Rad Protein Assay was used to determine protein concentration. Protein extracts were then subjected to SDS-PAGE electrophoresis and transferred to Inmobilon-P PVDF membrane (Millipore, Bedford, US). Antibodies to PAR-1 (ab32611) and EPCR (P20) were used for specific binding followed by chemiluminiscent detection.

2.4.9 Immunohistochemistry

PAR-1 expression in fixed left ventricle paraffin sections was detected with anti-rat PAR-1 antibody ATAP2 using DAB and Mayers-Haematoxylin and Li_2CO_3. Negative control slides were prepared by preadsorption of the primary antibody with its immunizing peptide (100 fold molar excess) and by staining in the absence of the primary antibody.

2.4.10 Data Analysis

Analysis was conducted using pClamp 9 (Molecular Devices, CA, US) and Origin 6 (Microcal Softwares, Northampton, US) softwares. Figures were prepared using Origin version 6.0 (Microcal Software Inc, Northampton, MA, USA). Results were expressed as mean ± standard error (SEM).

Effect of APC on shortening

1. The amplitude of shortening following APC application was normalised to control (S_{APC}/ $S_{control}$). The time constant of relaxation of the shortening (τ) was calculated by fitting the average of 10 samples to a single exponential function for each condition.

2. For the effect of APC on cell shortening with time, the changes in the magnitude of shortening induced by APC were normalised to the control value obtained for each cell ($S_{control} - S_{APC}$)/ $S_{control}$ [256, 257]. Peak amplitude of shortening was plotted *vs.* time and fitted to an exponential function: $y = y_0 + A.e^{-x/\tau}$, where y_0 is the maximal amplitude of shortening and τ is the time constant. The inverse of the time constant was then plotted against APC concentration and fitted with a linear equation: $1/\tau = k_{(+1)}[APC] + k_{(-1)}$, where τ is the time constant of the apparent effect on the myocyte shortening, $k_{(+1)}$ is the association rate constant, and $k_{(-1)}$ is the dissociation rate constant. K_D is estimated by $k_{(-1)} / k_{(+1)}$. The biphasic dose-response curve was fitted to a log-logistic^2 function designed by Beckon *et al.* [258]

The K_D values calculated for both the positive and negative apparent effects on the shortening of myocytes, were used as an estimate of each respective EC_{50} to fit the data to the log-logistic^2 equation [258].

Effect of APC on $[Ca^{2+}]_i$

The ratio of the fluorescence signals was converted into free Ca^{2+} concentration using the equation $[Ca^{2+}]_i = (R-R_{min})/(R_{max}-R).\beta.K_D$. and calculated following calibration as previously described [259] where R is the ratio of fluorescence, R_{min} is the minimum ratio recorded in zero Ca^{2+} solution, R_{max} is the maximum ratio recorded in saturated Ca^{2+} solution, β = the ratio of the minimal to the maximal fluorescence elicited by stimulation at 380nM and K_D= the dissociation constant. R_{min} and R_{max}

were estimated to be respectively 0.43 and 1.93, β was calculated to be 7.8. K_D was calculated to be 0.196μM using the Invitrogen "Dissociation Constant (K_D) Calculator" [260]. For each cell, after achieving steady state, 10 consecutive transients in ECS were recorded as control, followed by exposure to different concentrations of APC. The readings were used to calculate an average to characterise intracellular Ca^{2+} handling. The parameters analysed included systolic and diastolic levels, transient elevation, time to peak amplitude of level, time constant of the decay of the transient [256].

2.4.11 Calculation of the association ($k_{(+1)}$) and dissociation constant ($k_{(-1)}$)

From left to right: (1) example of cardiomyocyte shortening increases recorded before and after the application of 5nmol/L APC, (2) changes in the amplitude of shortening (dots) plotted against time and fitted to a mono-exponential function (dashed line), (3) plot of the reciprocal of time constants calculated using the fitted data in (2) against the concentration of APC used. The equation used for the linear fit to calculate the dissociation and association constants is indicated above the graph in step (3).

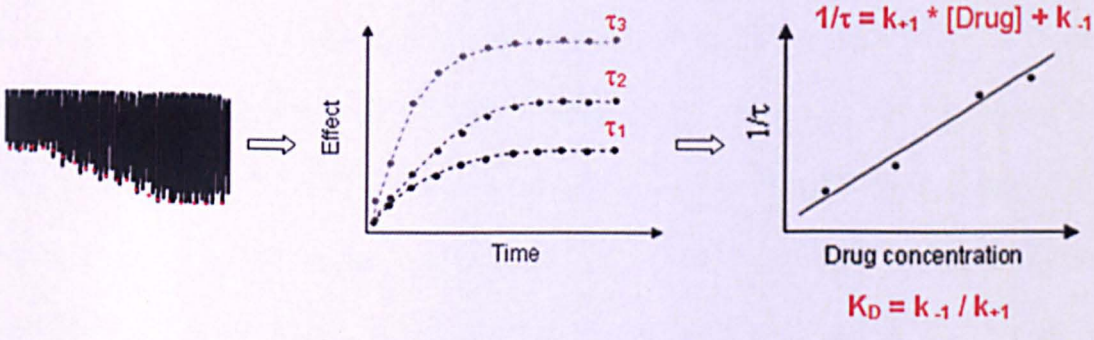


Figure 2.9 Stepwise Calculation of Association and Dissociation Constants
Linear regression fits of 1/τ against APC concentration were used to calculate K_D for ascending and descending phases of APC effect on dS.

2.4.12 Statistical Analysis

The paired Student *t*-test or the Mann-Whitney matched-pairs sign rank test was used when the normality test failed (Anderson-Darling). For comparison between more than 2 means, 1-way analysis of variance followed by multiple comparisons versus control group was undertaken using the Holm-Sidak method. For data that were not normally distributed, the Kruskal-Wallis test was used followed by Dunn's method for multiple comparisons. All statistical analyses were carried out using SigmaStat version 3.0 (Systat Software, London, UK) and $p<0.05$ was taken to denote statistical significance.

Chapter 3 The Effect of APC on HCAECs

3.1 Introduction

As highlighted in Chapter 1, the present literature supports the strong possibility of a direct action by APC on the heart. In relation to work from our group, APC can induce the release of MPs whose bound APC complexed to EPCR expresses proteolytic activity [91]. This translates functionally into anticoagulant, anti-inflammatory and cytoprotective properties. Our group in Liverpool have reported the release of APC-induced MPs whose bound functional APC confers anti-coagulant activity [91] . This data was obtained using human umbilical vein endothelial cells (HUVECs) and the mechanism of APC induced MP formation was shown to be dependent on the EPCR-APC-PAR-1 axis.

As previously described (above) APC signalling requires the co-proximity of PAR-1-EPCR. Cheng *et al* showed that APC infusion in to mice causes PAR-1 dependent down regulation of pro-apoptotic p53 and neuroprotection in an ischaemic brain model[261]. In this model however, only a fraction of PAR-1 colocalises with EPCR but the beneficial effects of APC were still observed at EPCR-deficient sites.

Our previous data could explain this benefit with APC induced MPs providing a means of circulating the EPCR-APC complex as shown below in Figure 3.1. The APC bound to EPCR on MPs would be in the right conformation to cleave PAR-1 on the cell surface. We also know now that rhAPC treated septic patients have significantly higher levels of circulating EPCR-MPs [60]. As APC treated patients derive systemic anticoagulation from both free and MP-bound APC, a strategy that only delivers the EPCR-APC complex by bioparticles could be promising.

Furthermore such results would have application to stroke and cardiovascular medicine.

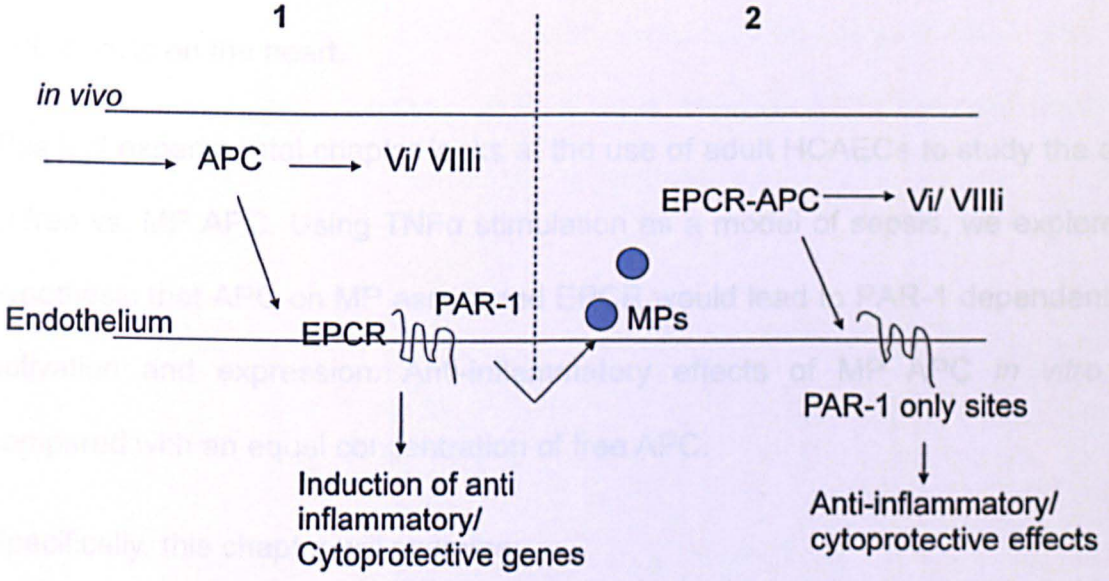


Figure 3.1 Schematic presentation of the potential value of MP APC vs. Free APC. Systemically infused APC will have the double hit effect of its initial anticoagulant and anti-inflammatory action followed by amplification of this by the effect of the MP-EPCR-APC performing the same, as shown by sections 1 and 2. This may explain the bleeding risk associated with APC treatment which is often a limitation to its use. Development of a technology to deliver APC in MP form could offer a more controlled response of some anticoagulation and cytoprotective benefits i.e. only actions shown in section 2.

Any effects of MP APC could also be distributed to relatively EPCR deficient sites such as the microvasculature, often a target of the endothelial damage caused by sepsis. The coronary endothelium is crucially involved in coronary blood flow and cardiac pathologies involving inflammation and coagulation such as atherosclerosis and thrombosis. HCAECs are therefore an ideal candidate for studying the effects of substances which may affect the upset equilibrium seen in sepsis. APC studies have

traditionally been performed using HUVECs and from evidence that local changes are important in influencing the development of vascular bed-specific thrombotic events [262] studies using HCAECs would be particularly relevant in understanding APC effects on the heart.

This first experimental chapter looks at the use of adult HCAECs to study the effects of free vs. MP APC. Using TNFα stimulation as a model of sepsis, we explored the hypothesis that APC on MP associated EPCR would lead to PAR-1 dependent gene activation and expression. Anti-inflammatory effects of MP APC *in vitro* were compared with an equal concentration of free APC.

Specifically, this chapter will examine:

1. EPCR expression on HCAECs

2. Ability of HCAECs to generate MPs

3. Ability of APC to induce signals on HCAECs

3.2 Results

3.2.1 Characterisation of HCAECs for EPCR

The presence of EPCR on HCAECs was demonstrated using RTPCR and FACS. EPCR could be clearly detected up to at least passage 12 of the cells however all further experiments were performed using passage 3-4.

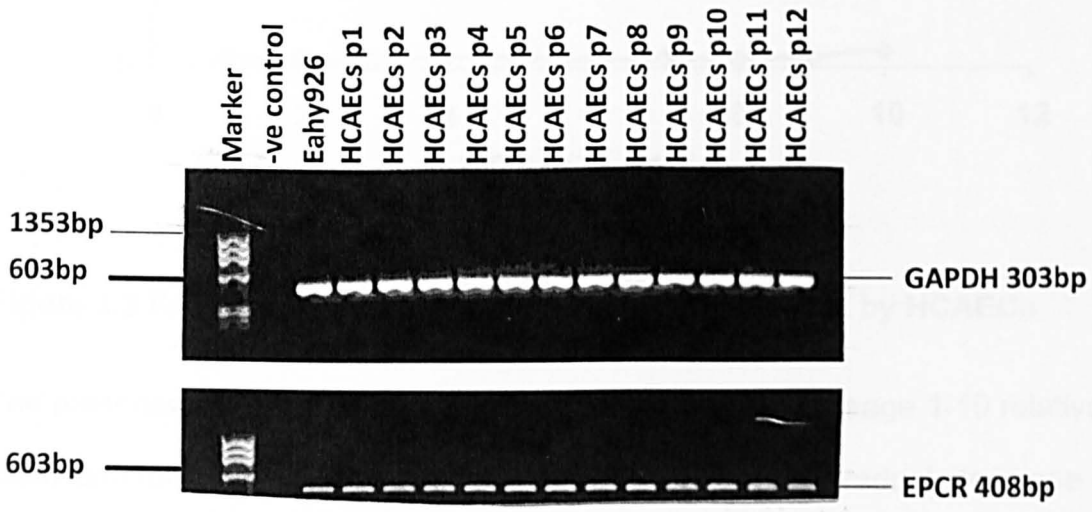


Figure 3.2 QPCR to demonstrate the expression of EPCR by HCAECs

The presence of EPCR RNA by RTPCR is shown for HCAECs passage 1-12 relative to the house keeping gene GAPDH. The first well represents the negative control in which no cDNA was included. The second well used Eahy926 cells as a positive control (previously confirmed to express EPCR).

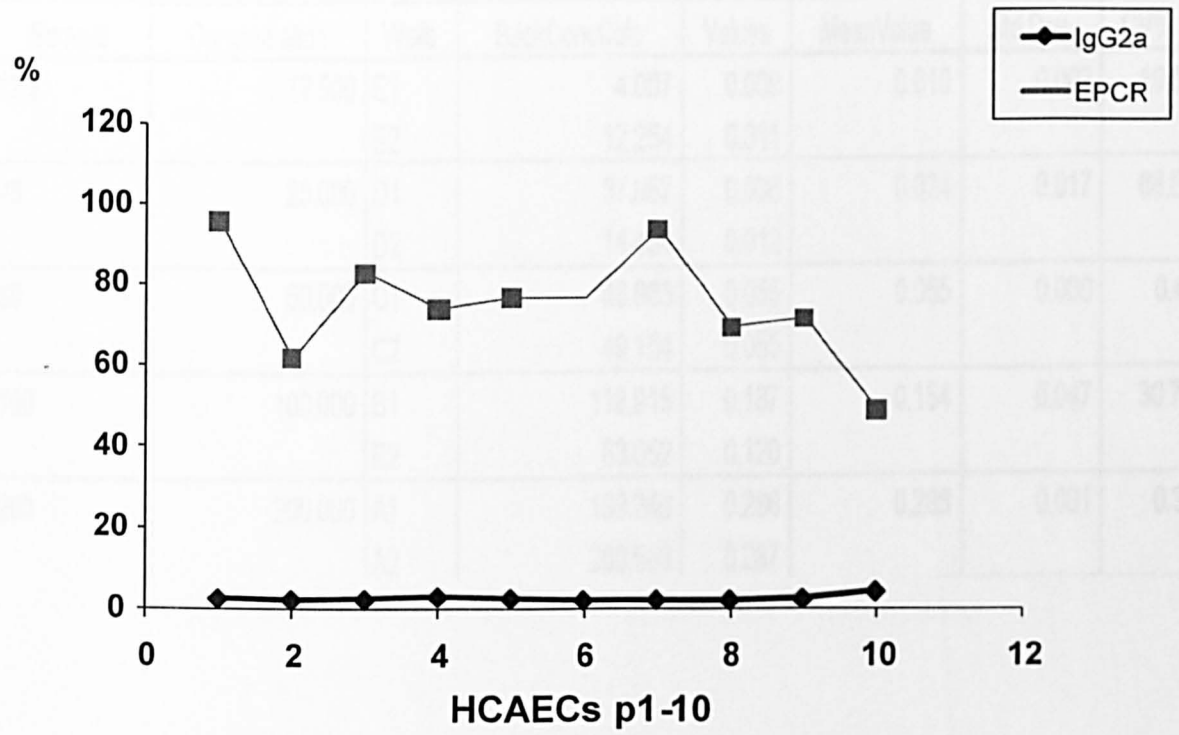


Figure 3.3 FACS to demonstrate the expression of EPCR by HCAECs

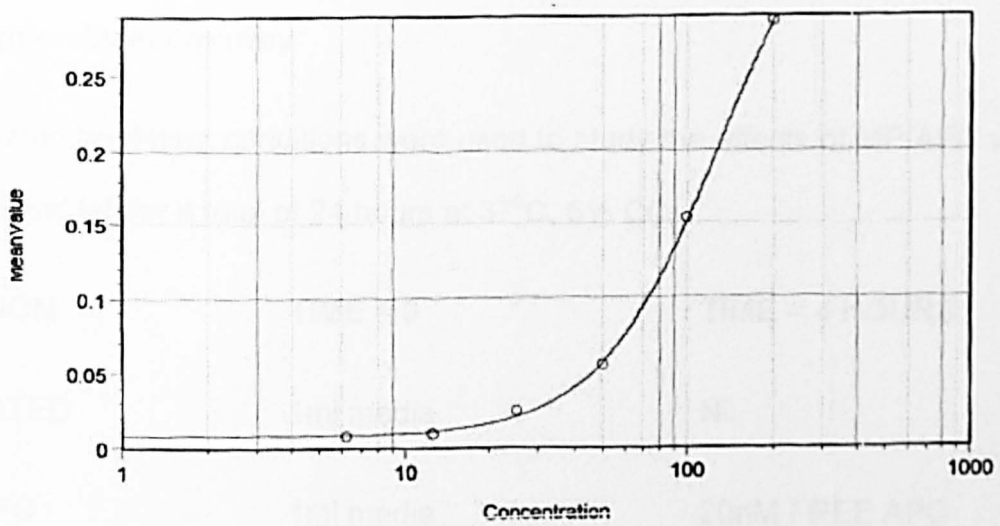
The presence of EPCR by FACS is shown for HCAECs passage 1-10 relative to the class control IgG2a.The expression of EPCR shows a gradual decrease as the passage of cells increases.

3.2.2 The Release of MP APC by HCAECs

Having demonstrated the expression of EPCR on HCAECs, confluent T25 flasks of HCAECs were induced overnight with 100nM APC to stimulate the production of MPs. MPs were harvested the following day and [MP APC] estimated. MP APC concentrations were calculated from a standard curve generated using dilutions of APC stock. A chromogenic substrate S2366 was added to an aliquot of MPs in buffer and read at 405nM on a spectrophotometer.

Sample	Concentration	Wells	BackConcCalc	Values	MeanValue	Std.Dev.	CV%
12.5	12.500	E1	4.007	0.009	0.010	0.002	19.6
		E2	12.254	0.011			
25	25.000	D1	37.057	0.036	0.024	0.017	68.6
		D2	14.434	0.012			
50	50.000	C1	48.963	0.055	0.055	0.000	0.4
		C2	49.154	0.055			
100	100.000	B1	118.915	0.187	0.154	0.047	30.7
		B2	83.052	0.120			
200	200.000	A1	199.246	0.286	0.286	0.001	0.3
		A2	200.563	0.287			

Figure 3.4 Exampletable of Free APC dilutions 12.5- 200nM prepared to create the standard curve (shown below). MP APC concentration calculated relative to standard curve Values



y = ((A - D)/(1 + (x/C)^B)) + D:	A	B	C	D	R^2
Std (Standards: Concentration vs MeanValue)	0.008	2.053	129.904	0.401	1

Samples

Sample	Wells	Values	Outliers	Result	MeanResult	Std.Dev.	CV%
Sa01	A3	0.153		100.010	100.010	0.000	0.0
Sa02	B3	0.274		186.276	186.276	0.000	0.0

Figure 3.5 Free APC standard curve (above) and estimation of MP APC concentration (below) For MP samples Sa01 and Sa02, these correlated with APC values of around 100nM and 200nM respectively as shown in the result column.

Having confirmed that HCAECs stimulated with APC release MP bound APC, the effects of MP APC versus free APC could be compared. The harvested MP samples were appropriately diluted to achieve a final concentration of 20nM APC to compare with 20nM free APC in the subsequent experiments.

3.2.3 The Effect of FREE APC vs. MP APC on PAR-1 dependent gene transcription by Microarray

The following treatment conditions were used to study the effects of MP APC versus free APC and left for a total of 24 hours at 37^0C, 5% CO_2.

CONDITION	TIME = 0	TIME = 4 HOURS
UNTREATED	1ml media	Nil
FREE APC	1ml media	20nM FREE APC
MP APC	1ml media	20nM MP APC
TNF	10ng/ml TNF	Nil
TNF + FREE APC	10ng/ml TNF	20nM FREE APC
TNF + MP APC	10ng/ml TNF	20nM MP APC

The cells were then washed, trypsinised and lysed for RNA isolation. The RNA from each condition was used to generate biotinilated cDNA probes used for the detection of other cDNAs on microarrays.

The effect of free APC 20nM was compared with an equal concentration of MP APC through their effects on a range of genes involved in inflammation and apoptosis including some already known to be modulated by free APC through PAR-1 cleavage. Microarrays were used with cDNAs of genes expressed on endothelial cells. The Endothelial Biology array from Superarray contained 96 genes expressed

on endothelial cells linked to inflammation, angiogenesis, apoptosis, permeability and coagulation (Figure 3.6).

ACE 1	ACE2 2	ADAM17 3	AGT 4	AGTR1 5	AGTR2 6	ALOX5 7	ANGPT1 8
ANGPT2 9	ANXA5 10	BAX 11	BCL2 12	BCL2A1 13	BCL21 14	CASP1 15	CASP10 16
CASP3 17	CASP6 18	CCL2 19	CCL20 20	CCL5 21	CDH5 22	CFLAR 23	CHGA 24
COL18A1 25	CPB2 26	CRADD 27	CSF2 28	CSF3 29	CX3CL1 30	EDN1 31	EDN2 32
EDN3 33	EDNRA 34	EDNRB 35	ENPEP 36	F3 37	FGF2 38	FLT1 39	FLT4 40
FN1 41	ICAM1 42	ICAM2 43	ICAM3 44	IFNB1 45	IL11 46	IL14 47	IL15 48
IL1B 49	IL3 50	IL6 51	IL7 52	IL8 53	ITGA5 54	ITGAV 55	ITGB1 56
ITGB3 57	KDR 58	MMP1 59	MMP14 60	MMP2 61	MMP9 62	NOS2A 63	NOS36 64
OCLN 65	PECAM1 66	PLA2G4C 67	PLAT 68	PLAU 69	PLG 70	PTGIS 71	PTGS2 72
RIPK1 73	SELE 74	SELL 75	SELPLG 76	SERPINE1 77	SOD1 78	TEK 79	TFPI 80
TFPI2 81	THBD 82	THBS1 83	TIMP1 84	TNF 85	TNFAIP3 86	TNFRSF10C 87	TNFRSF10D 88
TNFRSF1B 89	TNFRSF6 90	TNFSF10 91	TNFSF6 92	VCAM1 93	VEGF 94	VWF 95	XDH 96
PUC18 97	PUC18 98	PUC18 99	BLANK 100	BLANK 101	BLANK 102	GAPD 103	GAPD 104
PPIA 105	PPIA 106	PPIA 107	PPIA 108	RPL13A 109	RPL13A 110	ACTB 111	ACTB 112

Figure 3.6 Microarray layout table with gene symbol and position information for 112 genes including housekeeping genes

Chemiluscent detection was used to interpret the arrays. The hybridised membranes were incubated with streptavidin alkaline phosphatase which binds specifically to the biotin in the probe. The AP was then detected by CDP-Star substrate which gives a signal that can be visualised by a digital image analyser.

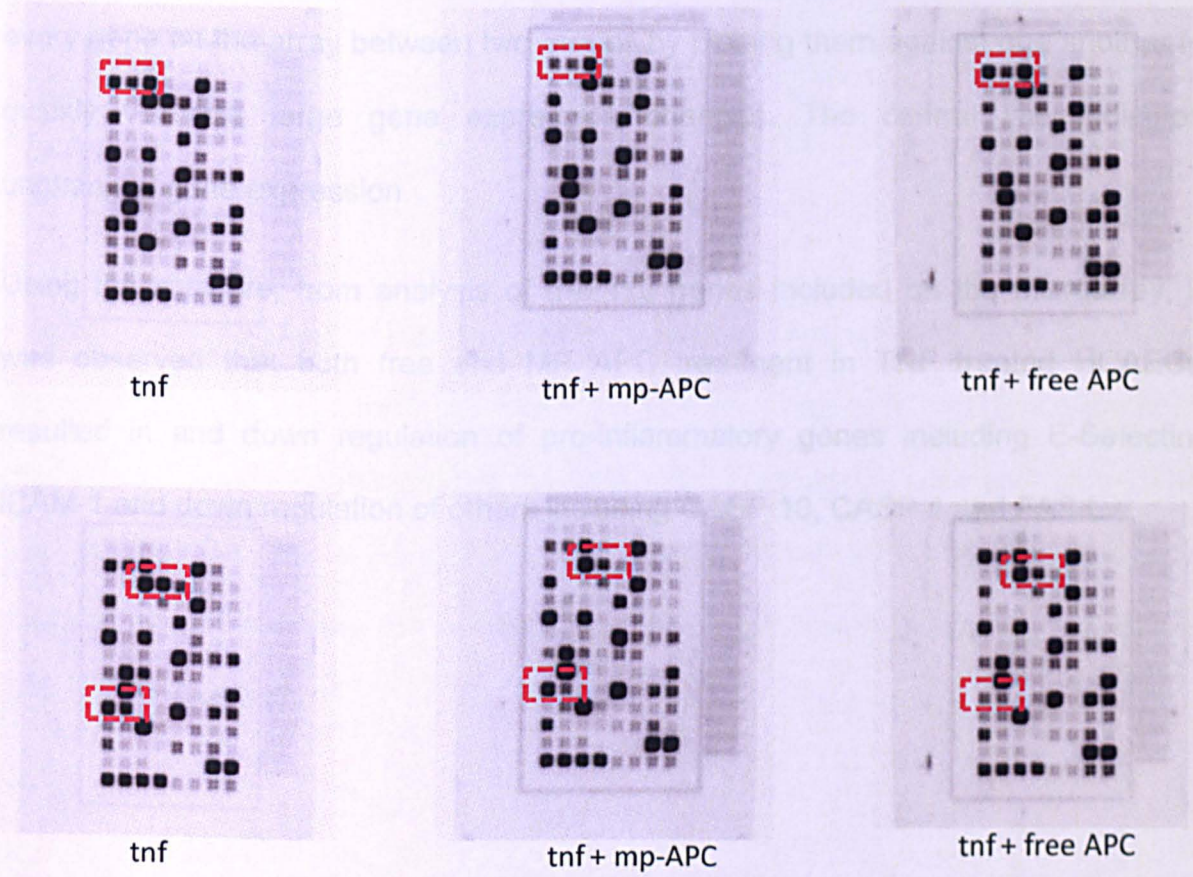


Figure 3.7 Microarray Bitmaps for HCAECs exposed to different stimuli. The spots of hybridisation were quantified using the GeArray software using median background subtraction. Treatment conditions are given below each Bitmap. The red boxes show gene positions where changes in expression can be directly visualised

and correlated with the gene layout table (above). For top 3 Bitmaps changes for ACE, ACE2 and ADAM17 highlighted, bottom 3 Bitmaps, changes for BAX, BCL2 and BCL2A1 (top) and RIPK1, SELE and SELL (bottom) shown. Up-regulation of expression was represented by darkness of specific Bitmap squares.

The integrated intensity values could then be represented using the Microarray Scatter plots and analysis of gene regulation under different conditions performed using the software provided. The scatter plot compared the normalized expression of every gene on the array between two groups by plotting them against one another to quickly visualize large gene expression changes. The central line indicates unchanged gene expression.

Using the software, from analysis of the 112 genes included on the microarray, it was observed that both free and MP APC treatment in TNF treated HCAECs resulted in and down regulation of pro-inflammatory genes including E-Selectin, ICAM-1 and down regulation of others including CASP 10, CASP 1 and FAS L.

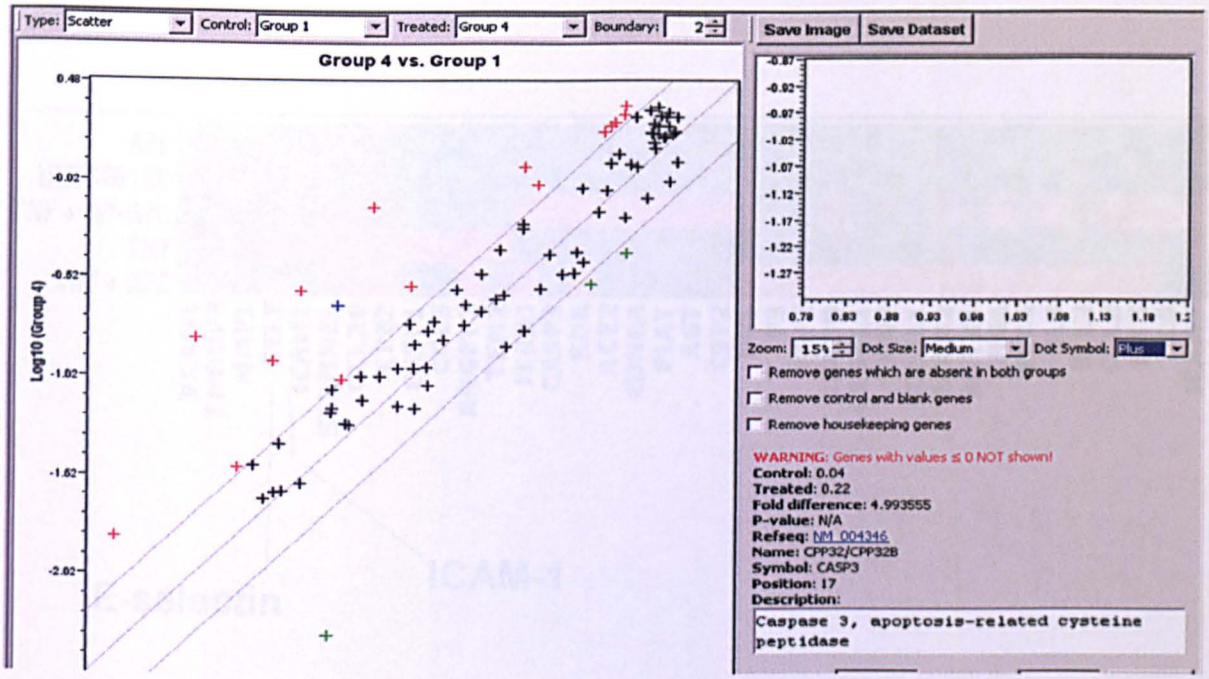


Figure 3.8 Microarray scatter plot example changes in gene regulation shown for conditions of Untreated vs. TNF (Group 4 vs. Group 1). The boundary lines (fold regulation cut-off) were set at 1.5 and then experimental conditions compared e.g. TNF vs. TNF plus APC. The software exports lists of genes whose expression changes are greater than the selected boundary. Red represents an up regulation, green down regulation.

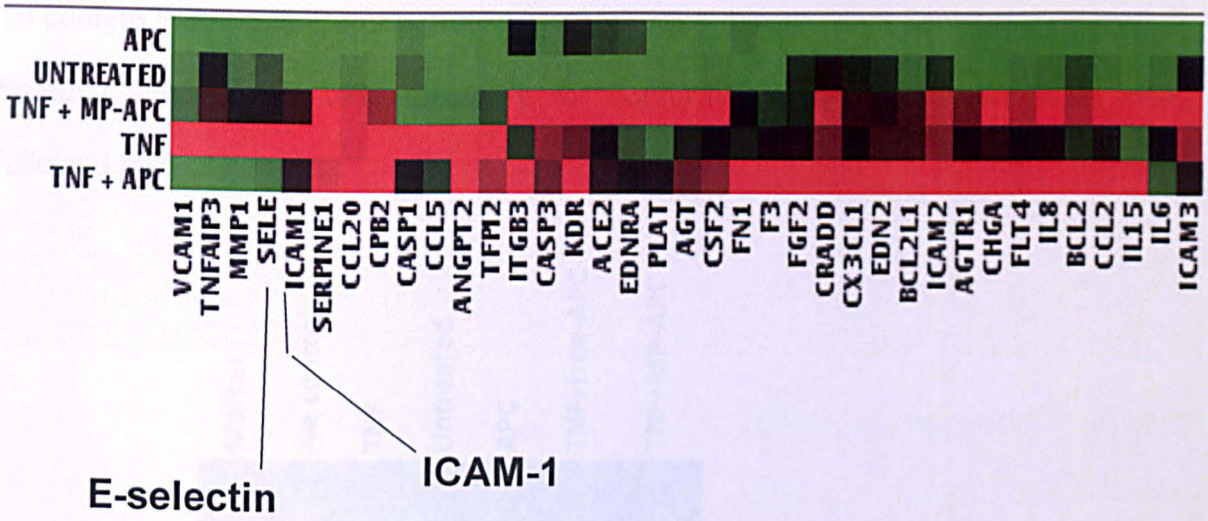


Figure 3.9 Microarray heat map example The heat map provides a graphical representation of fold regulation expression data between two groups overlaid onto the PCR Array plate layout using the same colour key. E-Selectin and ICAM-1 are highlighted to show the up regulation seen with TNF treatment which is reduced by the presence of APC, free and MP APC.

3.2.4 Confirmation of microarray results by Q-PCR

To confirm the results demonstrated by the arrays, target pro-inflammatory genes E-Selectin and ICAM-1 were selected for confirmation by semi quantitative PCR, followed by real time PCR.

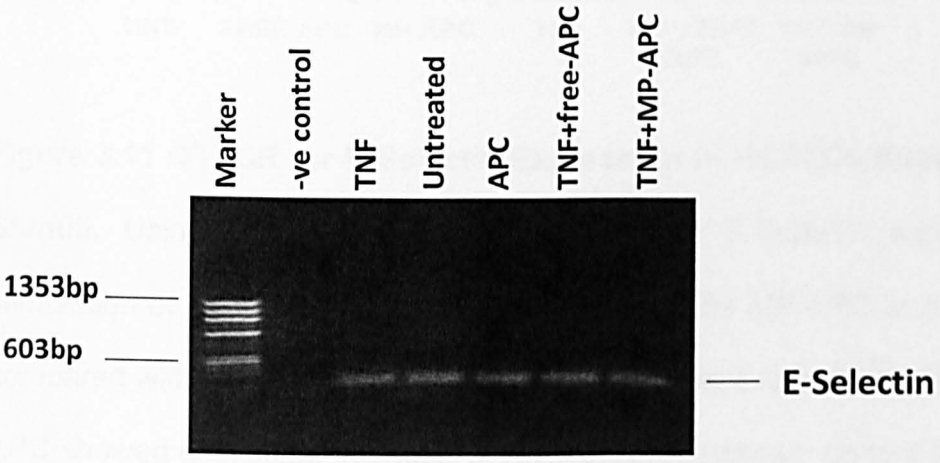


Figure 3.10 Gel electrophoresis for E-Selectin. Qualitative expression of E-Selectin for the treatment conditions shown above the gel

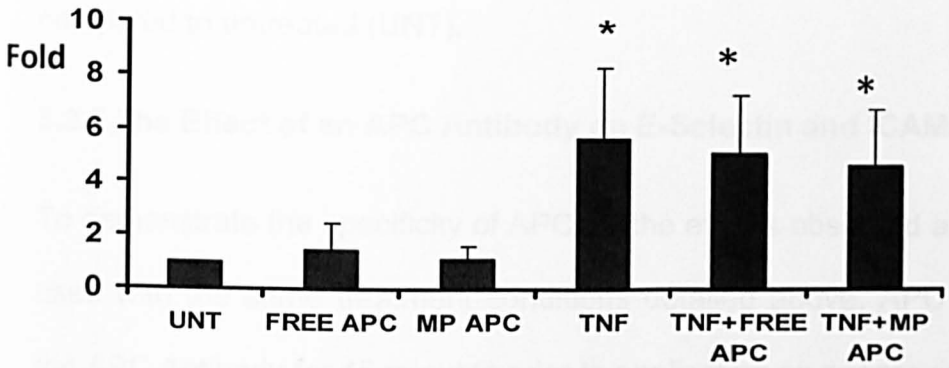


Figure 3.11 QTPCR for E-Selectin Expression in HCAECs Exposed to Different Stimuli. Using QTPCR the effect of APC on E-Selectin expression following stimulation of HCAECs with TNF showed a trend for MP APC to be more protective compared with free APC but no statistical significance. However, TNF with or without APC showed significant increase of E-Selectin expression up to 8 fold, *ANOVA test $p<0.05$ when compared to untreated (UNT).

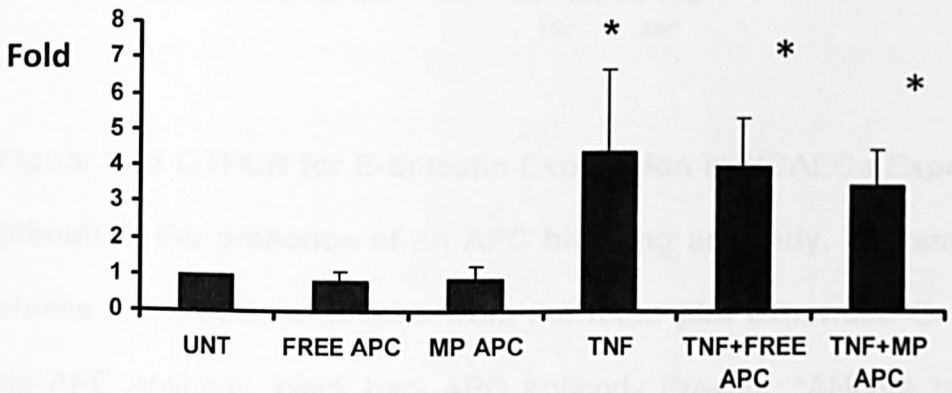


Figure 3.12 QTPCR for ICAM-1 Expression in HCAECs Exposed to Different Stimuli

Using QTPCR the effect of APC on ICAM-1 expression following stimulation of HCAECs with TNF showed a trend for MP APC to be more protective compared with free APC but no statistical significance. However, TNF with or without APC showed

significant increase of ICAM-1 expression up to 7 fold, *ANOVA test $p<0.05$ when compared to untreated (UNT).

3.2.5 The Effect of an APC Antibody on E-Selectin and ICAM-1 Expression

To demonstrate the specificity of APC for the effects observed an APC antibody was used with the same treatment conditions detailed above. APC was incubated with the APC Antibody for 45 minutes prior to application as per previous conditions.

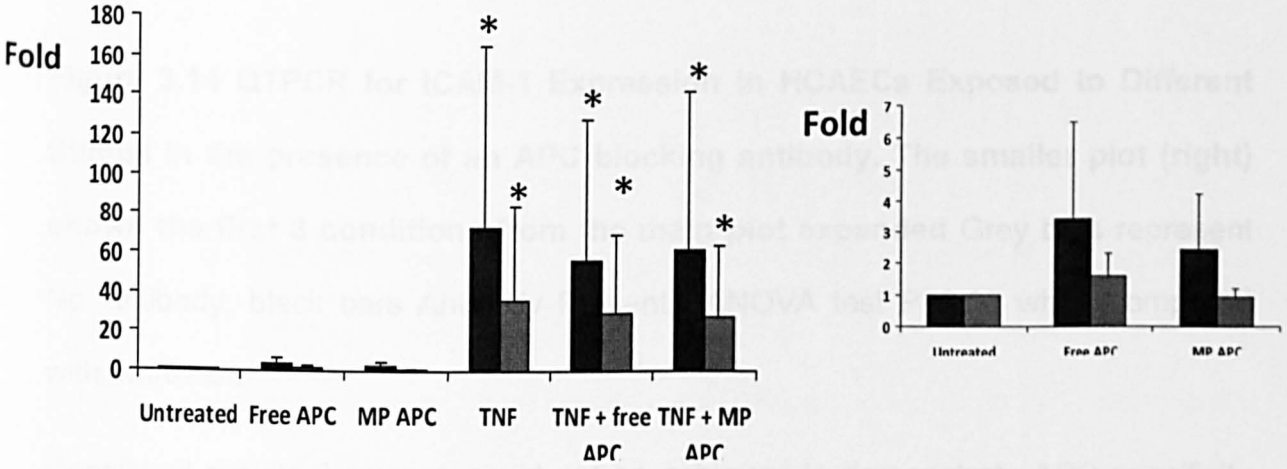


Figure 3.13 QTPCR for E-Selectin Expression in HCAECs Exposed to Different Stimuli in the presence of an APC blocking antibody. The smaller plot (right) shows the first 3 conditions from the main plot expanded Grey bars represent No APC Antibody, black bars APC Antibody Present. *ANOVA test, $P<0.05$ when compared with untreated.

The effect of free and MP APC on E-Selectin expression appears to be abrogated in the presence of the APC antibody suggesting the effects were APC specific. A similar effect could not be shown for ICAM-1 and for both molecules there was significant variation in results.

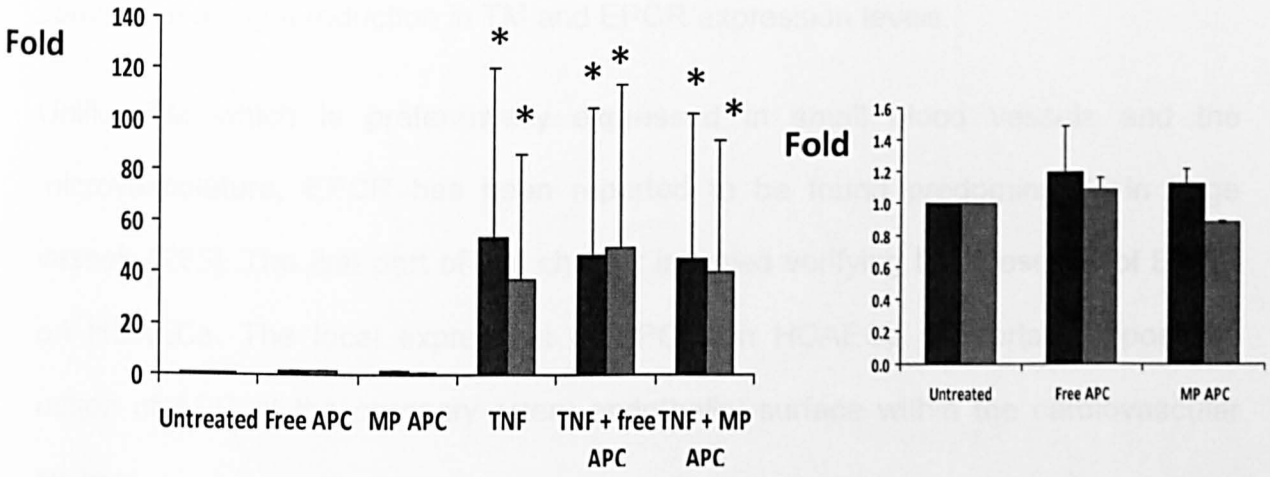


Figure 3.14 QTPCR for ICAM-1 Expression in HCAECs Exposed to Different Stimuli in the presence of an APC blocking antibody. The smaller plot (right) shows the first 3 conditions from the main plot expanded Grey bars represent No Antibody, black bars Antibody Present. *ANOVA test $P<0.05$ when compared with untreated.

Consistent results, however, could not be achieved to demonstrate APC specificity using blocking antibodies and there was a large degree of variation. Also, in Figures 3.13-14 above, an effect was seen with TNF and the antibody. The specificity of APC could not be consistently shown using boiled APC or the PAR-1 blocking antibody (data not shown).

3.3 Discussion

In severe sepsis, the host response leads to generalized dysfunction of the endothelium [263]. Under resting physiological conditions, PC is continuously activated to maintain an anticoagulant endothelial surface. This resting balance is shifted in favour of a prothrombotic state as tissue factor expression is increased in

response to cytokine release [264] and at the cell surface PC activation is compromised by a reduction in TM and EPCR expression levels.

Unlike TM which is preferentially expressed in small blood vessels and the microvasculature, EPCR has been reported to be found predominantly in large vessels [265]. The first part of this chapter involved verifying the presence of EPCR on HCAECs. The local expression of EPCR on HCAECs supports the possible action of APC at the coronary artery endothelial surface within the cardiovascular system.

In contrast to HUVECs, the primary cell line more frequently used for studying *in vitro* effects of APC, HCAECs have the advantage of representing adult cells directly found at the interface of coronary vessels and the smooth muscle of the heart. The phenotype of endothelial cells is suggested to change in the disease state relative to healthy conditions and may show differences between vascular beds. Accordingly, Hooper et al, showed that dependent upon their vascular bed origins and cell type, endothelial cells can demonstrate different responses to apoptosis[266]. This could be plausibly extended to the process of coronary inflammation especially as this has relevance to the development of atherosclerosis and the pathogenesis of coronary heart disease.

In this study we proved the hypothesis that HCAECs induced by APC could release MP bound EPCR-APC. This offers further support for the expression of functional EPCR on HCAECs. In comparative terms, APC in complex with MP-EPCR was found to be at least effective as free APC in the induction of PAR-1 signals responsible for the inhibition of cell inflammation and apoptosis. Furthermore, in the regulation of E-Selectin and ICAM expression, which are typically increased in

inflammation, a non-significant trend was observed suggesting it to be more effective than free APC in inhibiting HCAEC inflammation.

A limitation to the work in this chapter relates to the cell model, which only gives an impression of the *in vivo* setting. We found variability in the primary cells, especially in modelling the effects of sepsis.

TNF treatment was used in this regard. In the pathophysiology of sepsis, LPS is usually felt to be the initial endotoxic stimuli. This then triggers the release of inflammatory mediators such as IL-1and TNF. TNF and IL 1 activate neutrophils and endothelial cells and all activated cells release further inflammatory mediators [267]. As LPS represents the early stages of microbial injury, TNF relates more to the propagation of the host response to infection; i.e. sepsis. Moreover, this would be the clinical stage when APC would be considered for treatment in severe sepsis.

A further rationale for the choice of TNF in our experiments relates to work, for example from Wu *et al*, [106]. They compared changes in endothelial cell gene expression profiling in the response of different cultured cells to thrombin, TNF and LPS. The focus was on 5 target genes: Egr-1, TF, PDGF-A, ICAM-1 and u-PA. With HPAECs, TNF induced a 4.4 fold induction of ICAM-1 mRNA with maximum levels at a concentration of 2.5ng/ml at 2 hours. The effect of LPS was much lower. Using VSMCs, TNF resulted in a 3.5fold induction of ICAM-1 mRNA with maximum levels at 2.5-10ng/ml at 1-2 hours. LPS induced a 2.5 fold induction with maximum levels at <100ng/ml at 1-2hours. There would appear to be no benefit of studying LPS over TNFα.

Another experimental variable relates to timing of treatment conditions and sample collection for gene transcription analysis. Su *et al* comment on the oscillatory

expression of genes in response to TNF stimulation and suggest time resolved measurements of gene transcription as the preferred methodology but this is beyond the scope of this study.

In conclusion, the novel findings in this chapter are that HCAECs express functional ECPR that can respond to APC stimulation to generate MP-EPCR. APC in free or MP form can confer anti-inflammatory and cytoprotective effects on to HCAECs but this could not be convincingly demonstrated to reverse the pro-inflammatory effects of TNFα or specifically attributed to the singular effects of APC.

Chapter 4 The Effect of APC on Cardiomyocytes

4.1 Introduction

The previous chapter addressed the relevance of the coronary endothelium with respect to the clinical observation that treatment with recombinant human APC improves the speed and likelihood of cardiac recovery. As cardiomyocytes are also relevant to cardiac performance, this chapter will directly explore their response to APC.

Generated signals from APC binding to EPCR to facilitate cleavage and activation of PAR-1 [72] translate into anti-apoptotic and endothelial barrier-protective effects, which have been shown to be physiologically relevant in experimental models of ischemic stroke [50, 268, 269]. This led us to hypothesise that APC could exert a direct effect on cardiomyocyte function via EPCR and PAR-1 activation. As previously stated, the possibility that the APC-EPCR-PAR-1 pathway may be relevant to myocardial function is supported by the finding that EPCR is cardioprotective in a septic mouse model [55]. Low-expressing EPCR mice had histochemical evidence of more severe heart damage.

When considering possible pathways for a potential mode of action for APC, a basic knowledge of cardiac contraction physiology is of value. Excitation–contraction coupling represents the process from electrical excitation of the myocyte to contraction of the heart, resulting in ejection of blood from the ventricles. Ca^{2+} is the central player responsible for effective contractile function [270].

During the cardiac action potential, depolarization activates voltage-gated membrane L-type calcium channels (LCC) allowing an inward flux of Ca^{2+} (I_{Ca}) through the sarcolemma. Ca^{2+} entry activates Ca^{2+} release channels (the ryanodine receptors; RyRs) located in the junctional face of the sarcoplasmic reticulum (SR), and thereby

induces calcium release from the SR (calcium-induced calcium release; CICR). The resulting rise in intracellular Ca^{2+} concentration facilitates binding of Ca^{2+} to the myofilament protein troponin C to switch on the contractile machinery.

For relaxation to subsequently follow, the intracellular Ca^{2+} must decline to allow Ca^{2+} to dissociate from troponin. Calcium transport out of the cytosol occurs through four pathways primarily; SR Ca^{2+}-ATPase, sarcolemmal Na^{+}/Ca^{2+} exchange, sarcolemmal Ca^{2+}- ATPase or mitochondrial Ca^{2+} uniport. SR Ca^{2+} ATPase (SERCA) pumps Ca^{2+} ions back into the SR, while the Na+/ Ca^{2+} exchanger (NCX) transports part of the released Ca^{2+} out of the cell. SR calcium release also contributes to calcium dependent inactivation of the L current I_{Ca}. This negative feedback is essential for diastolic refilling of the heart.

The quantitative importance of the various routes can vary between species. The activity of SR Ca^{2+} -ATPase is higher in rat ventricle compared with rabbit due to a greater concentration of pump molecules (8), and Ca^{2+} removal through Na^{+}/ Ca^{2+} exchange is lower.

The development of contraction force depends on $[Ca^{2+}]_i$ and $[Ca^{2+}]_{tot.}$ The two main mechanisms through which the strength of cardiac contraction is altered are;

1. changing the amplitude / duration of calcium transient
2. altered myofilament sensitivity to Ca^{2+}

Myofilament sensitivity is reduced by; acidosis, ischemia and Beta adrenergic activation and enhanced by; caffeine and some inotropic drugs.

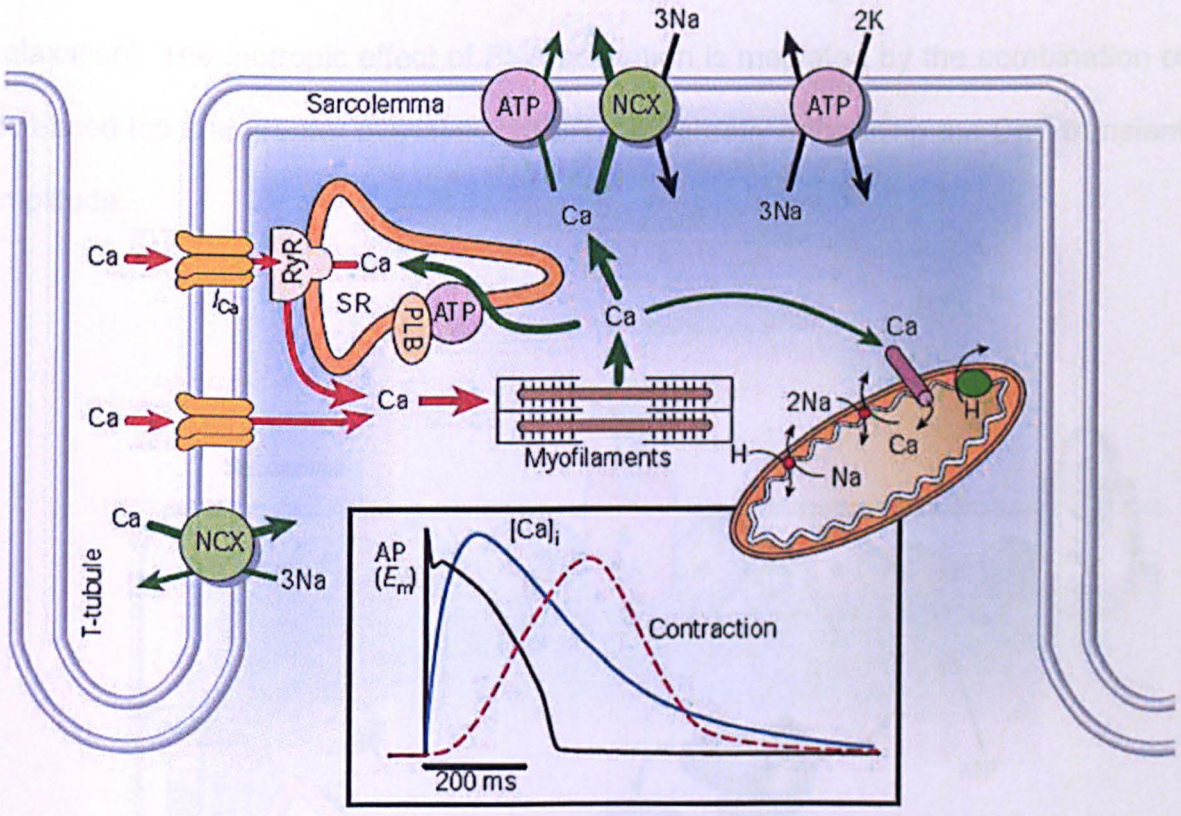


Figure 4.1 Ca^{2+} transport in ventricular myocytes. Inset shows the time course of an action potential, Ca^{2+} transient and contraction . NCX, Na+/ Ca^{2+} exchange; ATP, ATPase; PLB, phospholamban; SR, sarcoplasmic reticulum.

Modulation of calcium

Physiological sympathetic stimulation of the heart through beta adrenergic receptors increases contractions (inotropy) and accelerates relaxation plus calcium decline. Beta adrenergic receptor stimulation activates a GTP binding protein (Gs) stimulating adenylyl cyclase to produce cAMP to activate PKA. This kinase phosphorylates several proteins related to excitation-contraction namely, phospholamban, L-type channels, RyRs, troponin I and myosin binding protein C. Phosphorylation of phospholamban and troponin I speed up SR Ca^{2+} reuptake and dissociation of

calcium from the myofilaments respectively contributing to a lusitropic effect (relaxation). The inotropic effect of PKA activation is mediated by the combination of increased Ica and greater availability of SR Ca^{2+} greatly enhancing the Ca^{2+} transient amplitude.

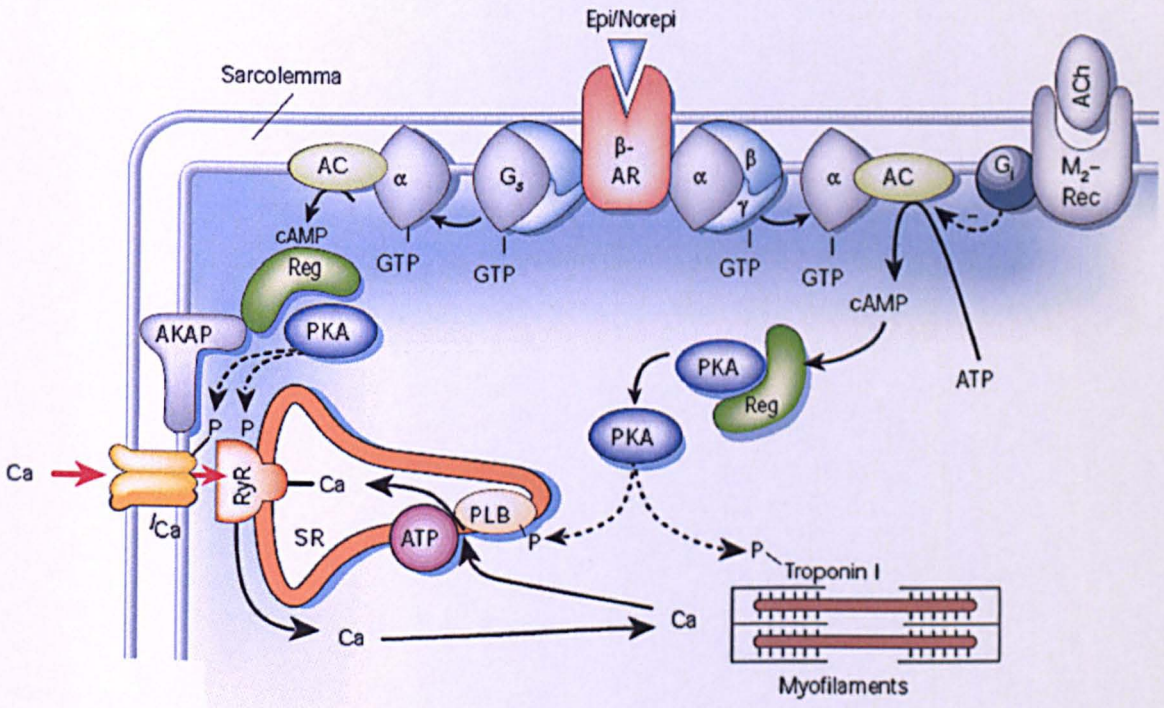


Figure 4.2 Activation and phosphorylation targets relevant to excitation–contraction coupling; Epi/Norepi represents Beta-adrenergic receptor activation. AC, adenylyl cyclase; ACh, acetylcholine; AKAP, A kinase anchoring protein; b-AR, b-adrenergic receptor; M2-Rec, M2-muscarinic receptor; PLB, phospholamban; Reg, PKA regulatory subunit; SR, sarcoplasmic reticulum

To address the broad hypothesis that APC PAR-1 may regulate cardiac function this chapter will specifically examine;

1. The effect of APC on cardiomyocyte shortening

2. The effect of APC on cardiomyocyte $[Ca^{2+}]_i$

3. The role of EPCR and PAR-1 in the action of APC

4.2 Results

4.2.1 The Effect of APC on Cardiomyocyte Shortening

Freshly isolated rat ventricular cardiomyocytes were treated with APC (0.1-50nM). Representative recordings of cardiomyocyte shortening amplitude (dS) in control state (left) and after increasing APC concentrations (right) are shown in Figure 4.3. Measurements were recorded and analysed using the p Clamp 9 software.

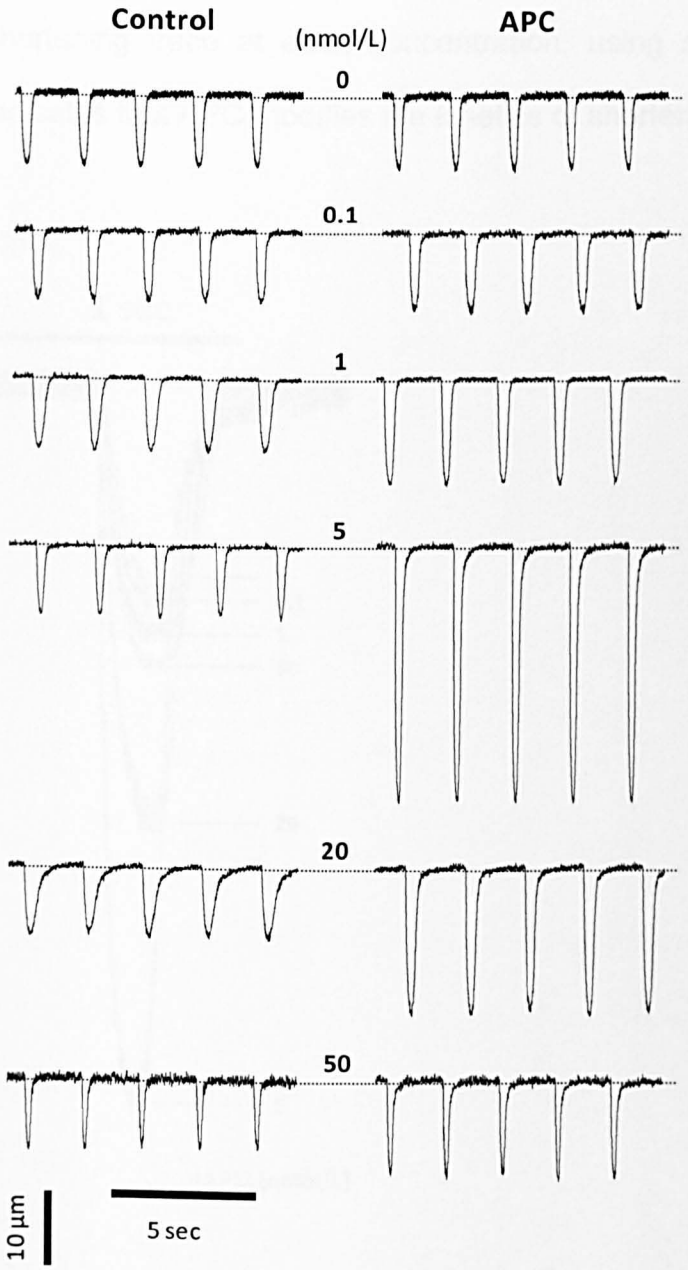


Figure 4.3 Representative records of cardiomyocyte shortening. Traces were obtained under control conditions (left) and increasing APC concentrations [0-50nmol/L] (right). Cardiomyocyte stimulation at 0.5Hz.

In addition to the APC effect on amplitude, superimposition of a representative shortening trace at each concentration, using similar time and amplitude scales indicates that APC modifies the kinetics of shortening as shown in Figure 4.4.

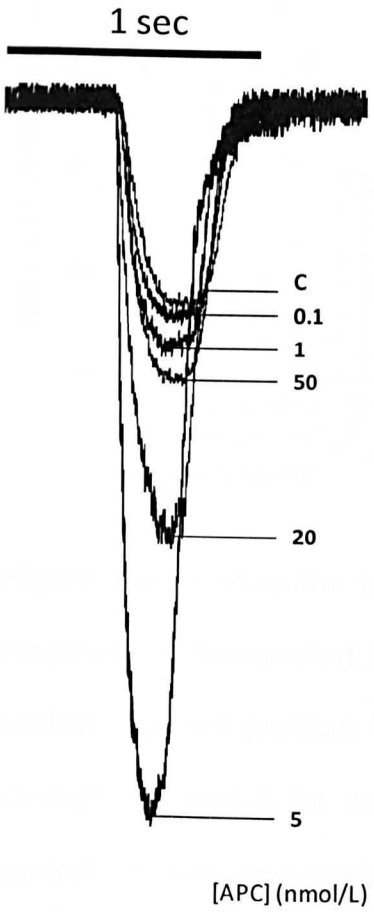


Figure 4.4 Superimposed single shortening recordings at different APC concentrations; normalised to control (expanded to the same scale).

As [APC] increased, we observed a bell-shaped concentration response curve with significant increases in peak shortening for concentrations of APC between 1-50 nmol/L shown in Figure 4.5. The maximal increase in peak shortening, 203.7 ± 28.0% relative to control (9.3 ± 0.4μm), was seen with 5 nmol/L APC (18.9 ± 2.4μm).

Increasing the concentration of APC beyond 5 nmol/L resulted in peak shortening values returning towards control levels.

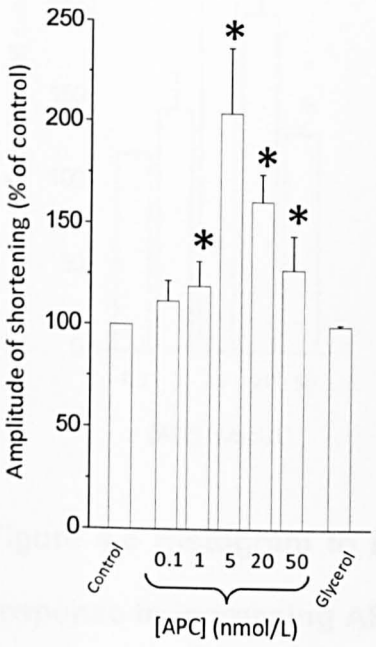


Figure 4.5 Histogram to show the mean amplitude of shortening (dS) in response to increasing APC concentrations; expressed as percentage of mean control, glycerol (vehicle buffer) was included as control. Data are expressed as mean±SEM; n=4–6 for each concentration. * indicates $p<0.05$ when compared to control (Students paired t-test).

Analysis of the maximum velocity of shortening (dS/dt) showed that APC significantly increased the speed of the shortening as shown in Figure 4.6 following a similar pattern to the effect on the amplitude of shortening. Again, the maximum effect on dS/dt (344.6 ± 59.4um/s) compared to control (142.5 ± 22.7) was observed at 5nmol/L APC.

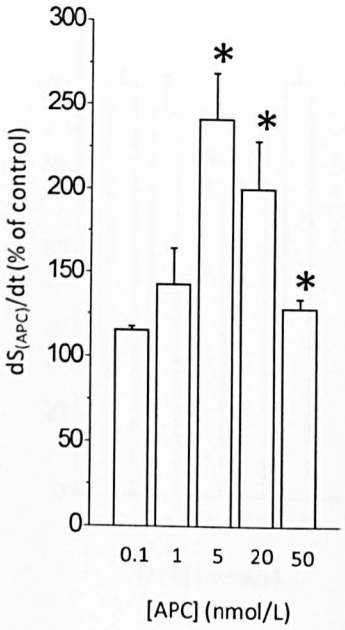


Figure 4.6 Histogram to show the maximum velocity of shortening (dS/dt) in response to increasing APC concentrations; expressed as percentage of control

This pattern of effect on dS/dt was supported by a similar modification of time to peak (TTP) (Figure 4.7) but the time constant of relaxation (Figure 4.8) was not significantly modified by APC as compared to control (169.2 ± 28.8).

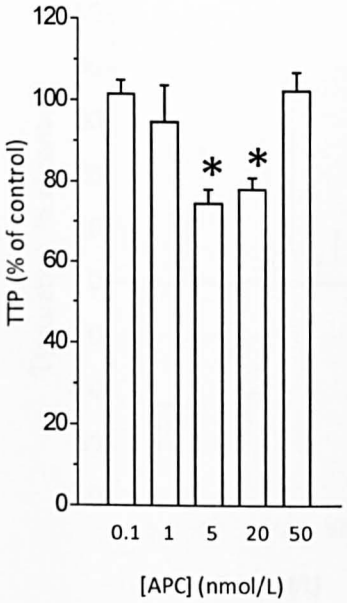


Figure 4.7 Histogram to show the time to peak (TTP) shortening in response to increasing APC concentrations; expressed as percentage of control. Data are expressed as mean±SEM; n=4–6 for each concentration. * indicates $p<0.05$ when compared to control (Students paired t-test).

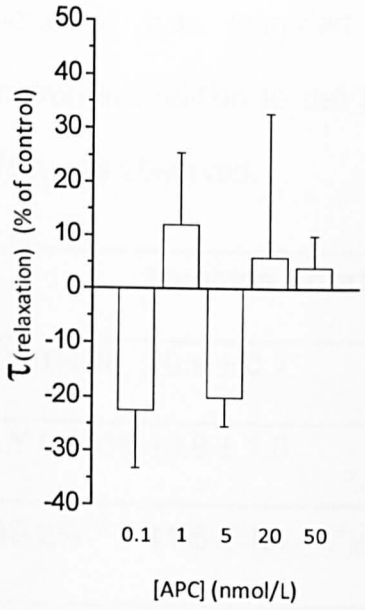


Figure 4.8 Histogram to show the time constant of relaxation (τ) in response to increasing APC concentrations; obtained by fitting traces to a mono-exponential function and expressed as percentage of control.

Shortening was recorded for cardiomyocytes placed in tyrode buffer solution (control) in addition to the APC vehicle buffers glycine and PBS to ensure that no effect was observed.

	Myocyte Shortening	dS/dt (um/s)	TTP (s)
CONTROL	10.9 ± 0.7	124.2 ± 17.0	258.2 ± 27.2
GLY 0.001%	10.8 ± 1.8	127.3 ± 7.9	273.5 ± 33.9
PBS 2%	11.5 ± 1.4	118.4 ± 23.6	267.4 ± 39.6

Table 4.1 Table to show control experiments for cardiomyocyte shortening; shortening, maximal velocity (dS/dt) and time to peak (TTP) recorded in control, with glycine and PBS (APC vehicle buffers). No significant change in shortening parameters was observed with APC vehicle buffers.

4.2.2 The Biphasic Response of Cardiomyocytes to APC

The effect of APC was further assessed with respect to the time needed to achieve a steady effect at each concentration. This was dependent upon APC concentration and the shortening response was observed to occur in 2 phases. Time required to reach a plateau effect on dS was assessed at each concentration to estimate K_D for each phase to enable modelling of the response. Traces of shortening recorded over time showed that time to achieve a plateau in dS decreased until 5nmol/L APC. Above this concentration, time to reach plateau increased with APC concentration (Figure 4.9).

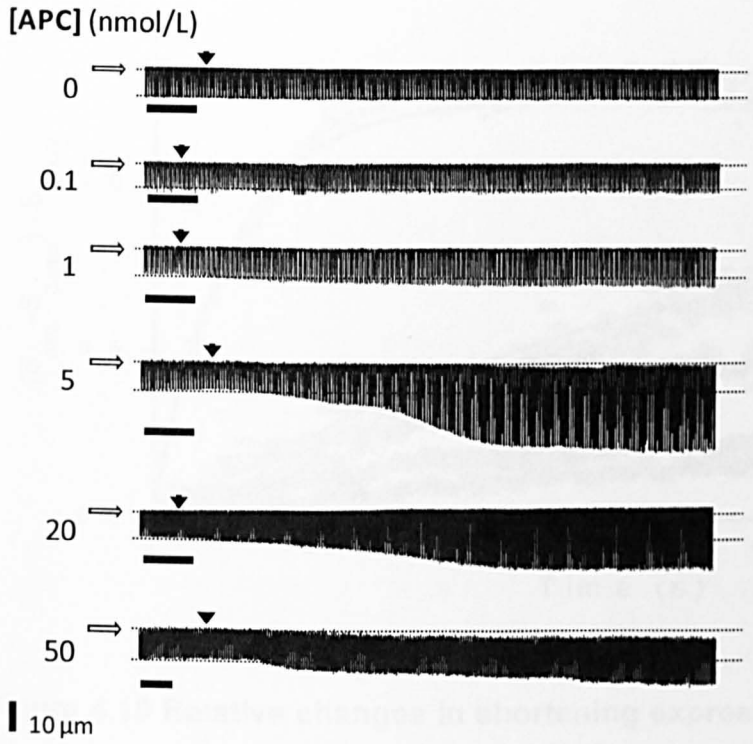


Figure 4.9 The biphasic modulation of shortening amplitude (dS) for increasing concentrations of APC 0-50nM. Representative records of APC effects on dS. The horizontal bar represents 1 min. Time of APC application is indicated by the black arrow.

To assess the biphasic response to APC, we applied the model designed by Beckon *et al* [258]. For each concentration used, the increase in shortening was normalised to control (recorded for one minute before the application of APC) and plotted *versus* time. The curves obtained were fitted to a mono-exponential function to obtain the time constants (T) for the effects of different concentrations of APC on myocyte contractility (Figures 4.10-11)

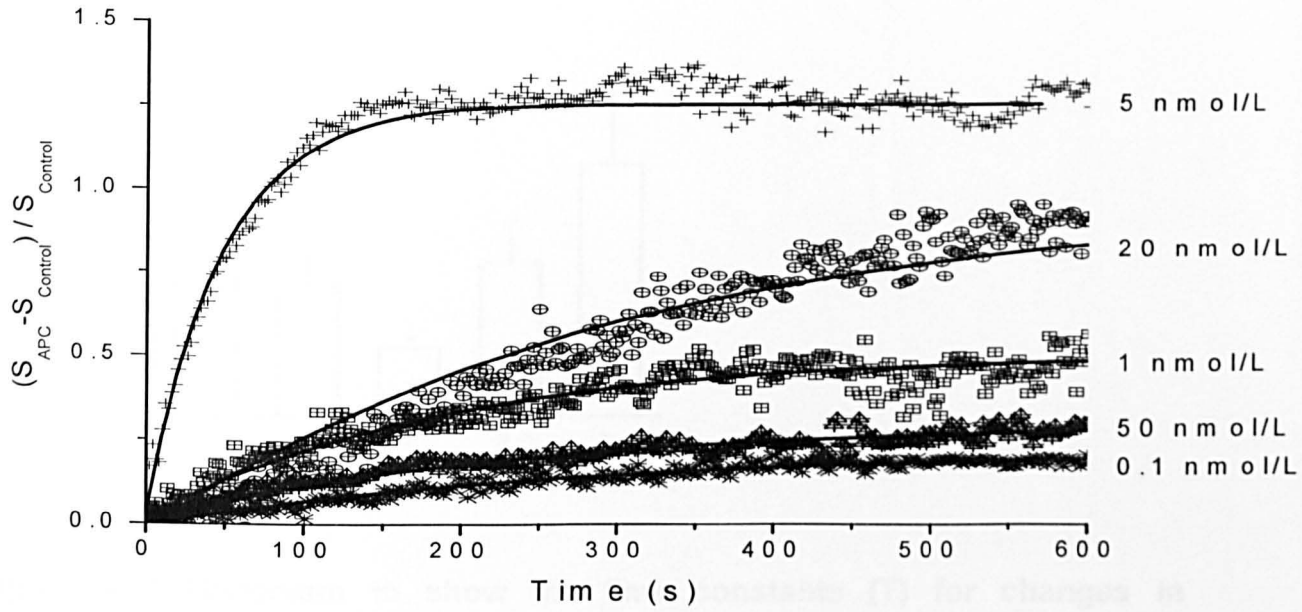


Figure 4.10 Relative changes in shortening expressed as a function of time for increasing APC concentrations. Each curve has been fitted to a single exponential function.

As the APC concentration increased to 5nmol/L, the effect on shortening developed more quickly (Figure 4.9). τ(S/dt) fell from 3.51 ± 1.06 min at 0.1nmol/L to 2.30 ± 0.50 min at 5nmol/L APC. However, at concentrations above 5nmol/L, the time needed to achieve a steady effect on shortening increased with a rise of τ(S/dt) to 4.52 ± 1.31 min at 50nmol/L APC.

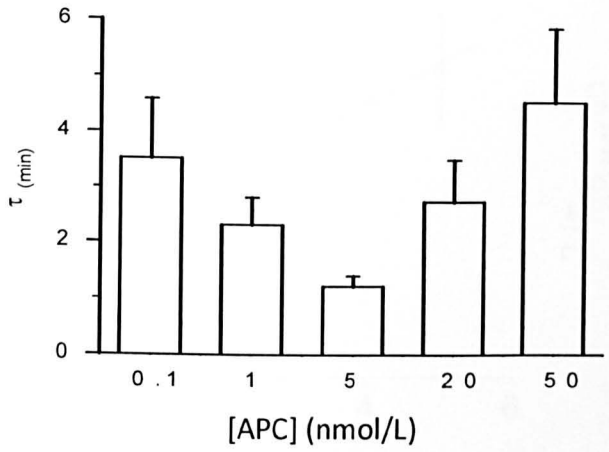


Figure 4.11 Histogram to show the time constants (T) for changes in shortening in response with increasing concentrations of APC.

To provide a model of the mechanism for the observed biphasic response, the *K*D was calculated from the time constants of the observed effects induced by APC on shortening. For APC concentrations between 0.1 and 5 nmol/L, a plot of the reciprocal of T *versus* concentration gave observed association (*k*+1) and dissociation (*k*-1) rate constants of 0.116 ± 0.025 nmol/L.min-1 and 0.327 ± 0.056 min-1 respectively (Figure 4.12). From these values, the K_{DUP} (*k*-1/*k*+1) for the first phase was calculated to be 2.82 nmol/L. For APC concentrations between 5 and 50nmol/L, the plot yielded observed association and dissociation rate constants of 9.190 ± 4.280 pmol/L.min-1 and 0.721 ± 0.167 min-1 respectively, giving a K_{DDN} of 78.3nmol/L.

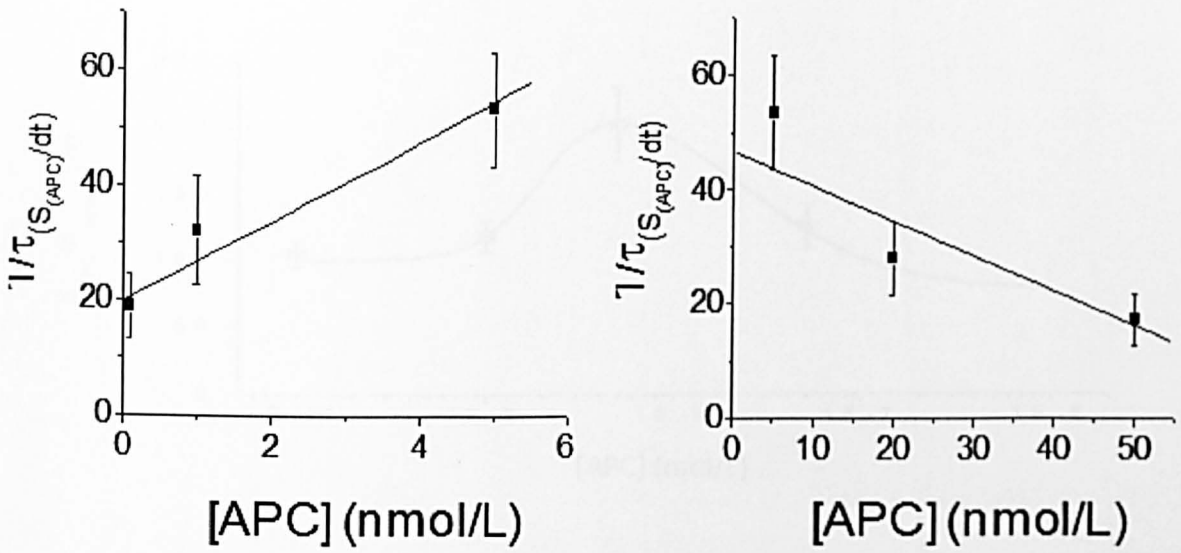


Figure 4.12 Linear regression fits of 1/T against APC concentration; these plots were used to calculate K_D for ascending and descending phases of the APC effect on shortening

Using calculated values for K_D as an estimation of EC_{50} for each phase, the concentration response was fitted to a log-logistic[2] function to model the biphasic response (Figure 4.13)[258]. This predicted that increasing APC concentrations could result in dS reaching 238.4±29.5% of control with a power of 2.58±0.47 and an EC_{50} of 1.99±0.36nmol/L (control 100%). The sharp rise of the upward slope suggests that 2 mechanisms may underlie the ascending phase of the dose response. In the descending phase, the model predicts that further increases in APC concentration could result in dS falling below control (77.79±7.67%) with an IC_{50} of 32.9nmol/L and power of 1.15±0.33.

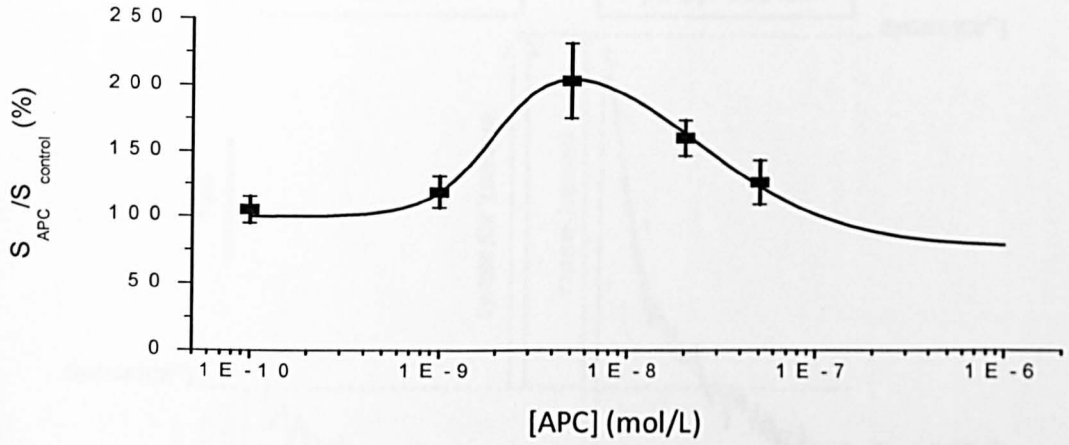


Figure 4.13 Relative changes in shortening in response to increasing APC concentrations, fitted to a log-logistic2 function. Data are expressed as mean±SEM; n=4–6 for each concentration.

4.2.3 Effect of APC on $[Ca^{2+}]_i$

The cycling of intracellular calcium $[Ca^{2+}]_i$ forms the basis of excitation-contraction coupling whereby a rise in $[Ca^{2+}]_i$ stimulates cardiomyocyte contraction.

Figure 4.14 shows a representative trace of the $[Ca^{2+}]_i$ recorded during cardiomyocyte electrical stimulation under control conditions (*left trace*), and after application of 750nmol/L of APC(APC) (*right trace*).(This supra-physiological concentration of APC was used as a test during preliminary experiments to detect any effect of APC on $[Ca^{2+}]_i$).

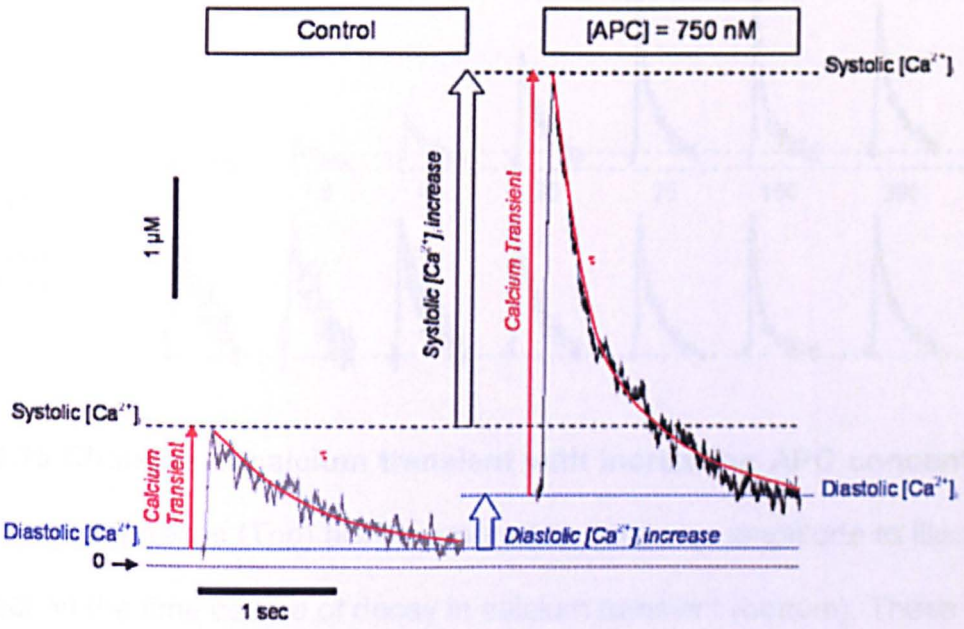


Figure 4.14 Example traces showing the effect of APC on $[Ca^{2+}]_i$ - preliminary data obtained using 750nM APC

The peak $[Ca^{2+}]_i$ (systolic) is indicated by dashed lines in the two traces to highlight the increase due to APC application. The resting $[Ca^{2+}]_i$ (diastolic) is indicated in both conditions by blue dotted lines and the increase induced by APC application is indicated in a similar fashion as for peak $[Ca^{2+}]_i$. The transient $[Ca^{2+}]_i$, resulting from the difference between the peak and the resting $[Ca^{2+}]_i$, in indicated in red for each condition. The vertical bar represents 1µmol/L variation of $[Ca^{2+}]_i$ and the level of $[Ca^{2+}]_i$ =0 is indicated with a black dotted line at the bottom of the graph. The horizontal bar represents 1 second.

Example traces showing changes in Calcium transient

Figure 4.15 below shows representative traces of calcium transients recorded following application of increasing APC concentrations (top).

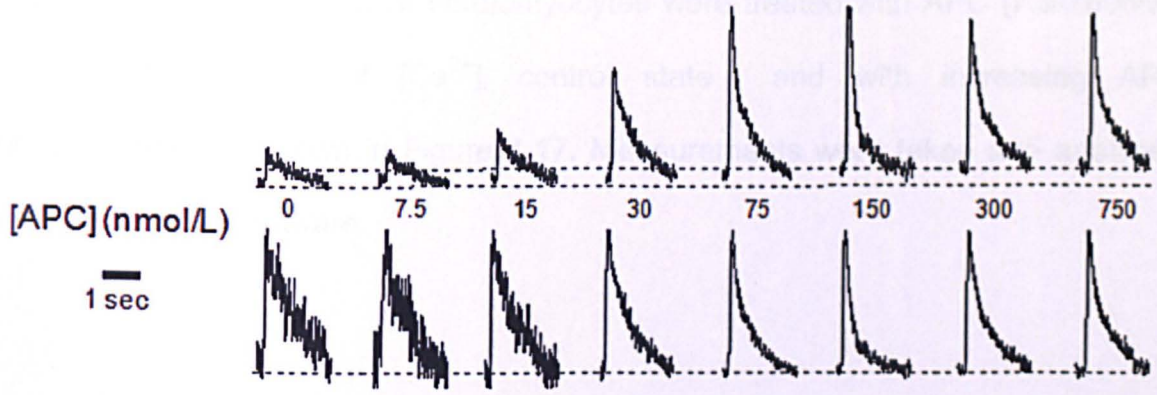


Figure 4.15 Changes in calcium transient with increasing APC concentrations. Calcium transient traces (Top) are normalised to the same amplitude to illustrate the APC effect on the time course of decay in calcium transient (bottom). These illustrate the observed effect on $[Ca^{2+}]_i$ reuptake i.e. the rate of decay increases with APC concentration.

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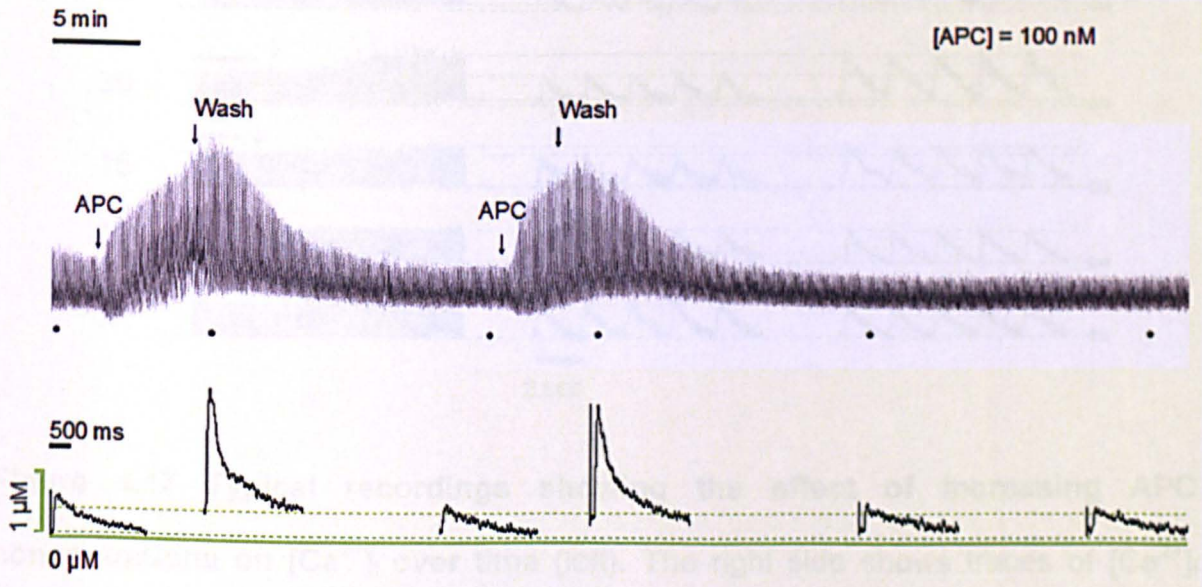


Figure 4.16 Example of APC induced calcium transient APC; application of APC followed by a 'wash' (perfusing with buffer solution) decreasing the effect of APC. Top trace expanded below to show representative individual transient traces (below).

Freshly isolated rat ventricular cardiomyocytes were treated with APC (7.5-750nM). Representative traces of $[Ca^{2+}]_i$ control state and with increasing APC concentrations are shown in Figure 4.17. Measurements were taken and analysed using the Ionoptix software.

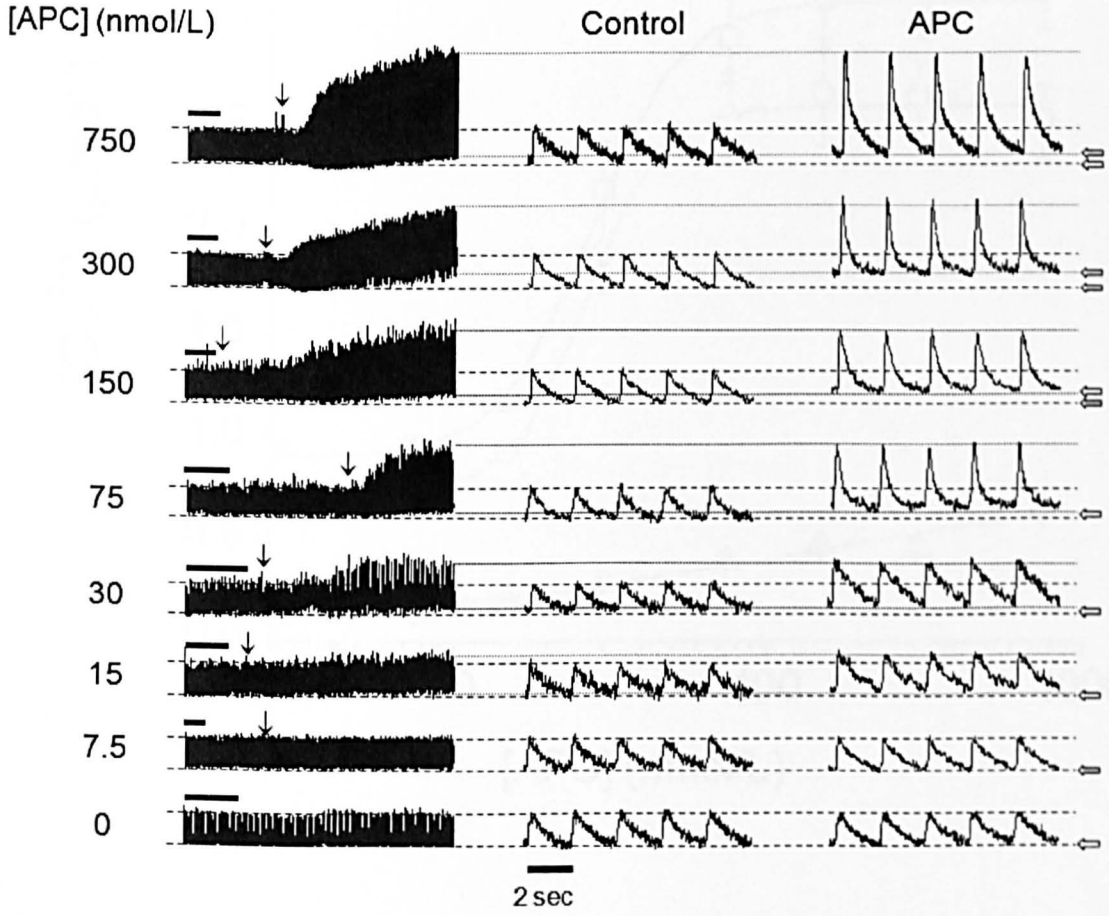


Figure 4.17 Typical recordings showing the effect of increasing APC concentrations on $[Ca^{2+}]_i$ over time (left). The right side shows traces of $[Ca^{2+}]_i$ expanded from recordings on the left using an adjusted timescale.

Increasing APC concentration induced a sigmoidal increase of both resting and peak $[Ca^{2+}]_i$ (Figure 4.18).

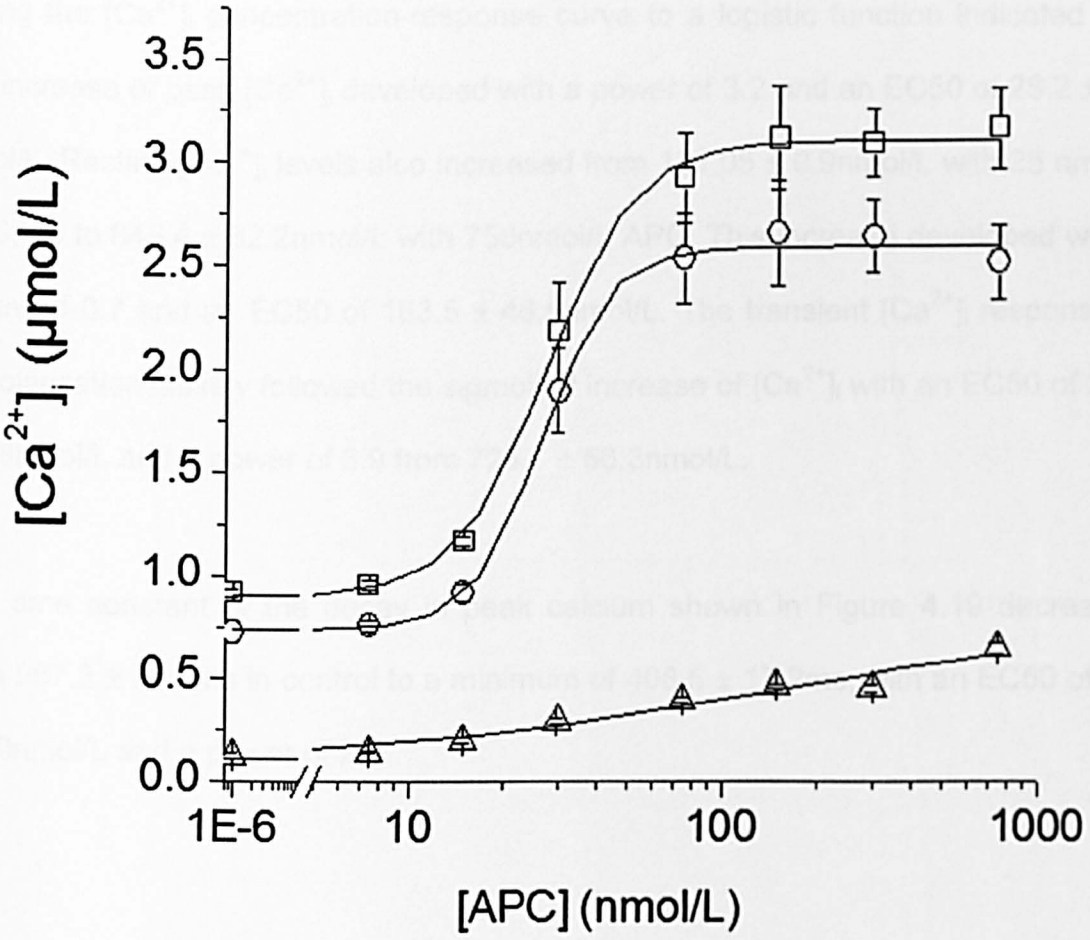


Figure 4.18 Graph to show changes in resting, peak and transient $[Ca^{2+}]_i$ with increasing APC concentration. A sigmoidal increase of both transient (○) and peak (□) $[Ca^{2+}]_i$ was observed. Resting $[Ca^{2+}]_i$ (Δ) also increased with APC.

Peak $[Ca^{2+}]_i$ increased significantly from 916.8 ± 15.8 nmol/L at 15 nmol/L APC to 3164.0 ± 197.0 nmol/L with 750 nmol/L APC. The maximal effect was observed from ~100 nmol/L of APC.

Fitting the $[Ca^{2+}]_i$ concentration-response curve to a logistic function indicated that the increase of peak $[Ca^{2+}]_i$ developed with a power of 3.2 and an EC50 of 28.2 ± 3.9 nmol/L. Resting $[Ca^{2+}]_i$ levels also increased from 121.05 ± 9.9nmol/L with 25 nmol/L APC, up to 645.4 ± 32.2nmol/L with 750nmol/L APC. This increase developed with a power of 0.7 and an EC50 of 163.5 ± 46.5nmol/L. The transient $[Ca^{2+}]_i$ response to depolarisation closely followed the sigmoidal increase of $[Ca^{2+}]_i$ with an EC50 of 26.4 ± 2.8nmol/L and a power of 3.9 from 725.7 ± 56.3nmol/L.

The time constant of the decay in peak calcium shown in Figure 4.19 decreased from 967.2 ± 70.7ms in control to a minimum of 408.5 ± 17.2ms, with an EC50 of 9.8 ± 1.0nmol/L and a power of 2.3

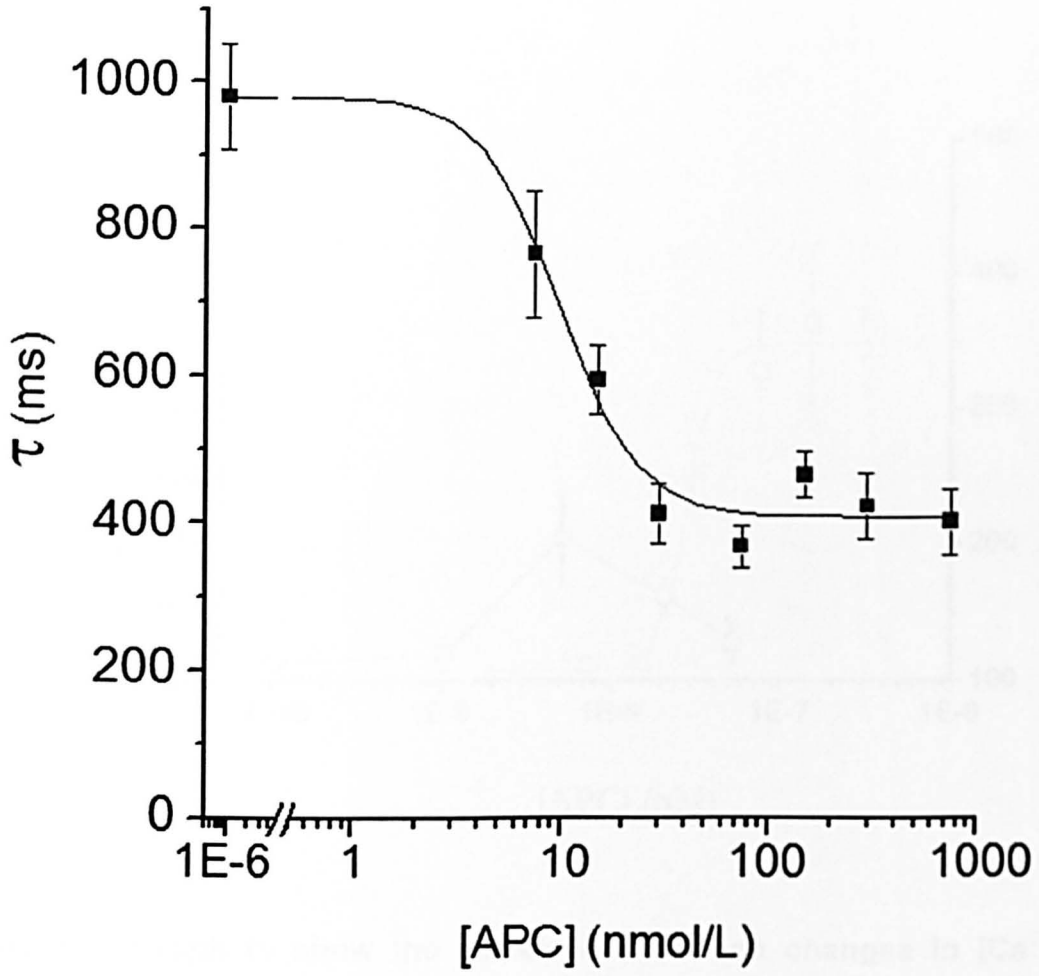
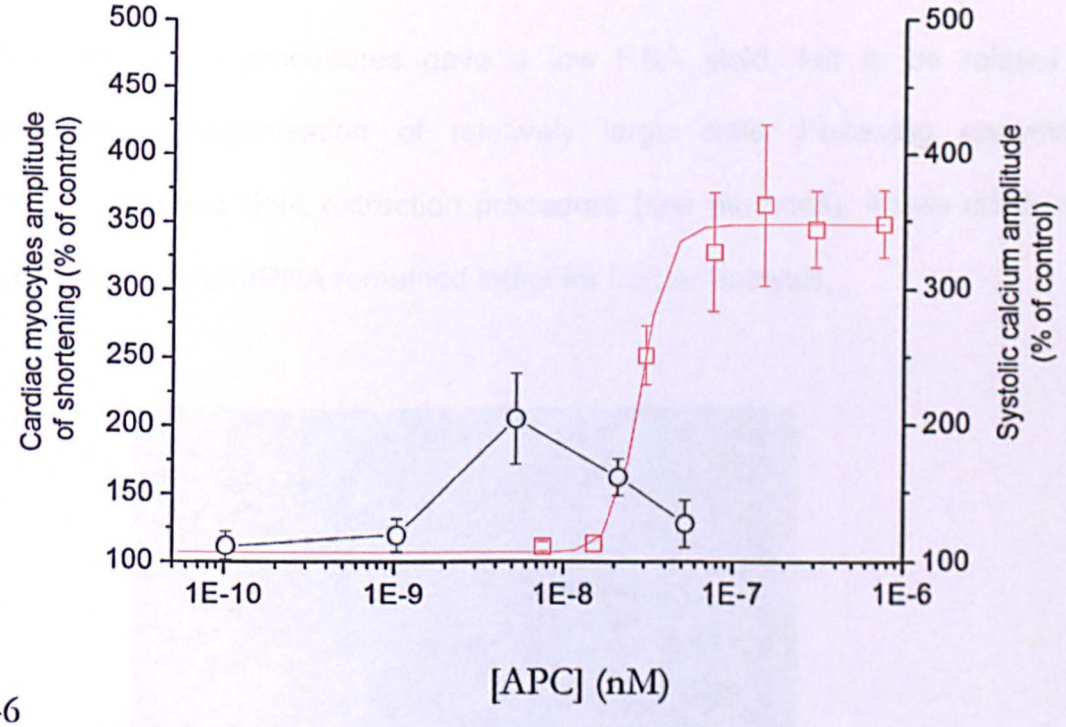


Figure 4.19 Change in the time constant of decay in peak $[Ca^{2+}]_i$ with increasing APC concentration

Combining results for the effect of APC on shortening and $[Ca^{2+}]_i$ provide information on possible mechanistic routes to explain our data. Figure 4.20 shows that the peak effect on shortening occurs at a relatively low concentration of Ca^{2+} however as the $[Ca^{2+}]_i$ increases the shortening effect becomes less pronounced. This suggests APC may be having an effect on myofilament sensitivity in particular reducing sensitivity up to a concentration of 5nM followed by the converse beyond this, up to 50nM APC.



n= 4-6

Figure 4.20 Graph to show the correlation between changes in $[Ca^{2+}]i$ and cardiomyocyte shortening relative to increasing APC concentration. Peak shortening (○) occurs before any significant rise $[Ca^{2+}]i$ suggesting that APC may increase myofilament sensitivity. Beyond 5nM APC, as the $[Ca^{2+}]_i$ increases, the effect on shortening falls suggesting a fall in myofilament sensitivity.

4.2.4 Role of PAR-1 and EPCR

Characterisation of cardiomyocytes for EPCR and PAR-1

Initial RNA extraction procedures gave a low RNA yield, felt to be related to ineffective lysis/homogenisation of relatively large cells. Following enzymatic isolation and a refined RNA extraction procedure (see methods), it was confirmed that the cardiomyocyte mRNA remained intact for further analysis.

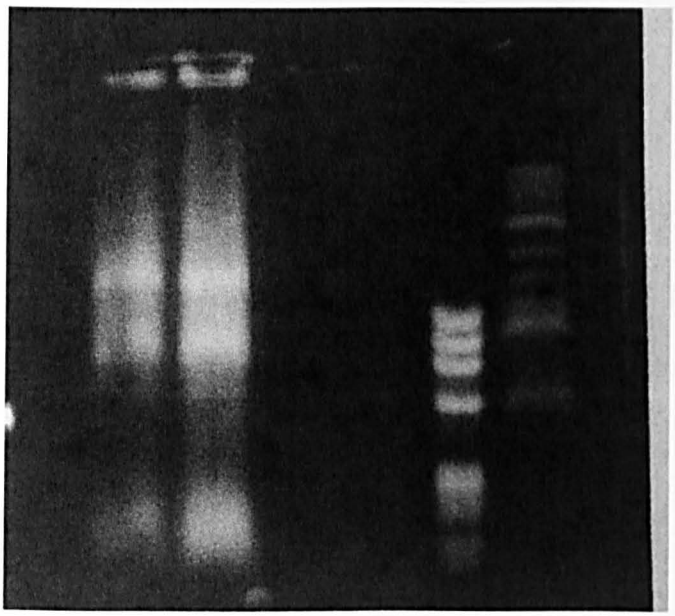


Figure 4.21 Specific ribosomal bands seen within RNA (left) extracted from cardiomyocytes (CM) confirming non-degradation of CM RNA following the enzymatic isolation procedure.

PAR-1 and EPCR mRNA were then detected by Q-PCR (Figure 4.22), and their presence was confirmed by sequencing.

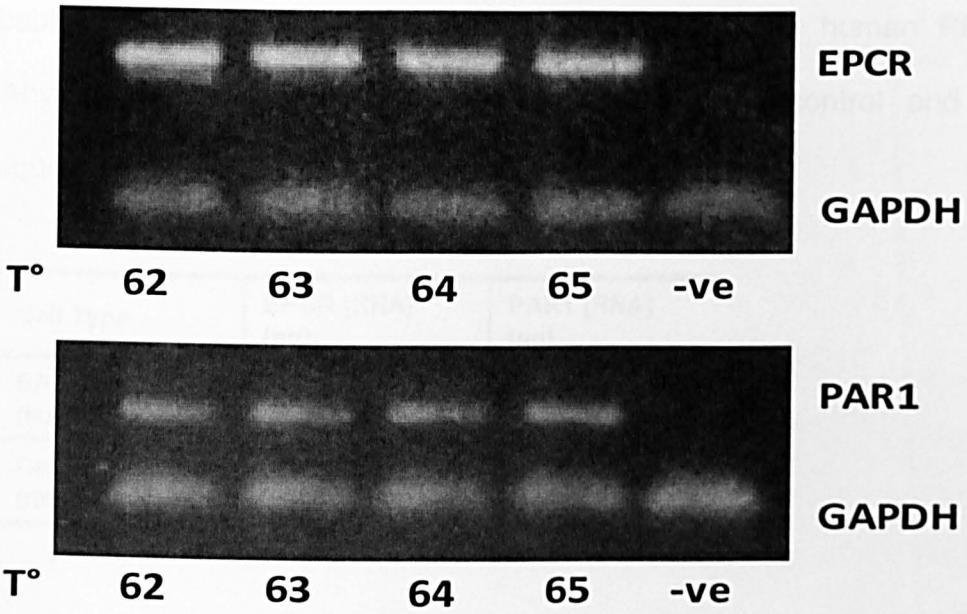


Figure 4.22 QPCR showing the expression of EPCR (top) and PAR-1 (bottom) on CMs. GAPDH used as a housekeeping gene. (Temperature conditions shown below each gel)

Amplification of EPCR and PAR-1 from cardiomyocytes was demonstrated using absolute quantification with external reference rat and human RNA, relative to EAhy926 (human endothelial cell line) as positive control and confirmed by sequencing (Table 4.2).

Cell Type	EPCR [RNA] (ng)	PAR1 [RNA] (ng)
EAhy926 (human)	109.1 ± 12.6	7.4 ± 3.4
Cardiomyocytes (rat)	9.8 ± 0.3	13.9 ± 3.4

Table 4.2 PAR-1 and EPCR mRNA expression on rat cardiomyocytes by qRT-PCR. Comparable cardiomyocyte expression of PAR-1, but reduced EPCR expression relative to human endothelial cell line EAhy926

Western blotting confirmed presence of both proteins (Figure 4.23). For PAR-1, a major band at the expected molecular weight (50kDa) was present in cardiomyocytes and human platelets (positive control) but not in KOLF cells (from PAR-1-knockout mice). A major band was demonstrated for EPCR at the same level as on EAhy926, which was absent in the negative control human prostate adenocarcinoma cell line (LNCaP).

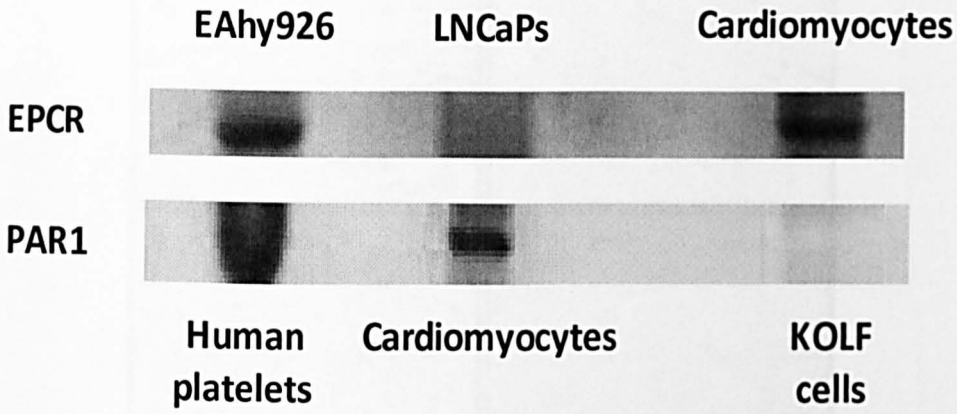


Figure 4.23 Western Blotting showing the expression of EPCR and PAR-1 on cardiomyocytes. EAhy926 and LNCaP are the positive and negative controls for EPCR, respectively. Human platelets and the PAR-1 knockout KOLF cell line are the positive and negative controls for PAR-1, respectively.

Membrane localisation of PAR-1 on the myocytes was demonstrated by immunohistochemistry. Preabsorption with an excess of PAR-1 antigen, or use of primary or secondary antibodies alone, did not produce any significant immunolabelling confirming the specificity of the result (Figure 4.24).

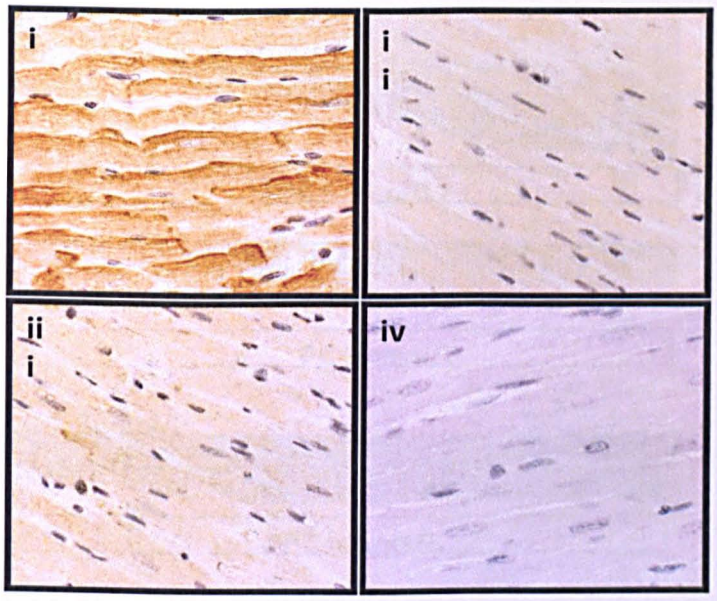


Figure 4.24 Immunohistochemistry showing the expression of PAR-1 on cardiomyocytes. Healthy rat left ventricular tissue was probed using sc-13503 (ATAP2). ii-iv show representative staining of controls: [i] membrane staining of PAR-1–dark brown, [ii] omission of secondary antibody, [iii] pre-incubation of PAR-1 antibody with excess blocking peptide, [iv] omission of primary antibody.

4.2.5 The Effect of EPCR/PAR-1 Blockade on APC induced Cardiomyocyte shortening

Anti-inflammatory and cytoprotective effects of APC have been shown to be mediated through the EPCR-PAR-1 axis. To examine if APC induced shortening effects were through an EPCR-PAR-1 signalling pathway, 5nmol/L APC was used in receptor-blocking experiments as this was the concentration that induced peak shortening responses. Sample traces are shown for the different blocking conditions (Figure 4.25).

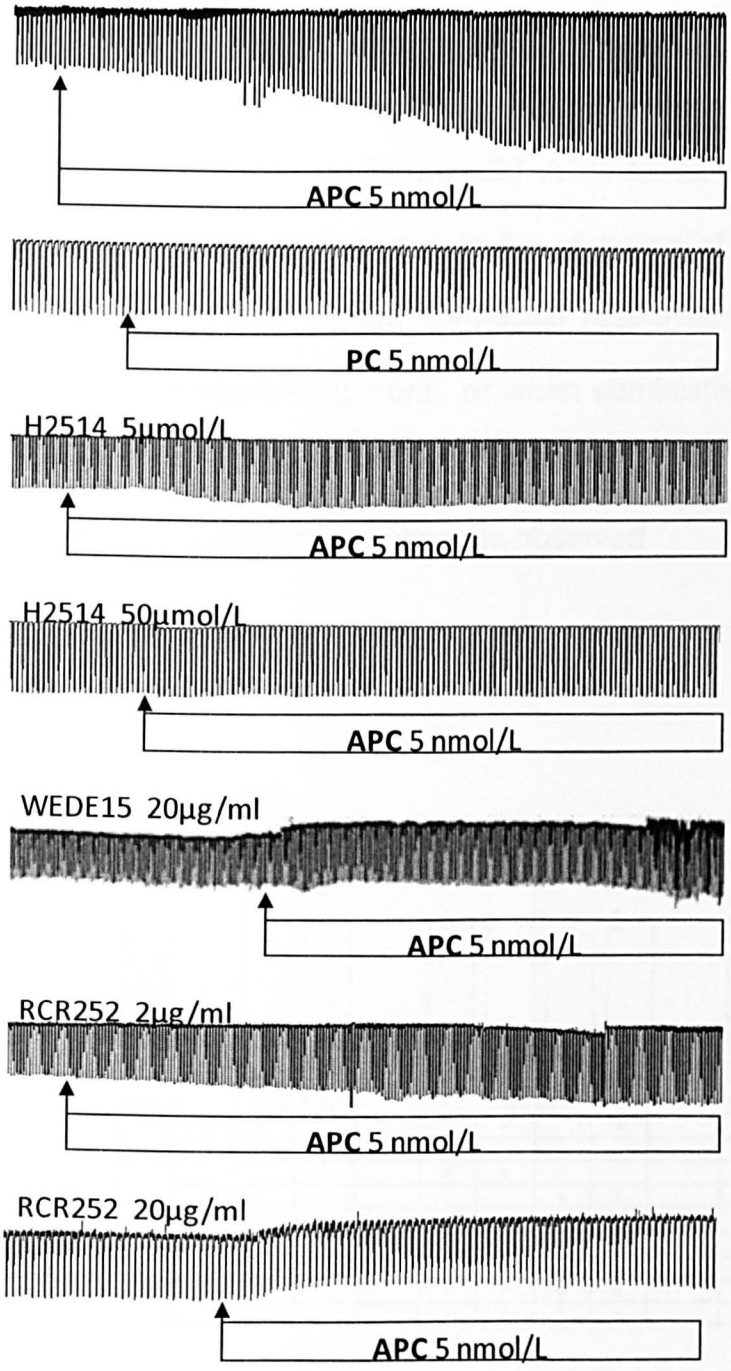


Figure 4.25 Representative traces to show the effect of PAR-1/ EPCR blockade on APC induced shortening. Pre-incubation with specific blocking agent to PAR-1 (H2514, WEDE15) or EPCR (RCR252) indicated above the trace. Application of APC indicated by arrow below trace. PC applied alone as further control (second trace).

Pre-incubation with blocking agents to PAR-1 (H2514, WEDE15) or EPCR (RCR252) significantly reduced the APC-induced effect on contractility as summarised in the histogram in Figure 4.26. A similar effect was seen on the kinetics of shortening development (Figure 4.26-28). Addition of zymogen protein C alone failed to induce an effect on the contractile response. Glycerol or PBS had no significant effect on shortening alone, or when administered with APC. These data further support a mechanistic role of PAR-1 and EPCR in APC-induced cardiomyocyte contraction for the changes observed.

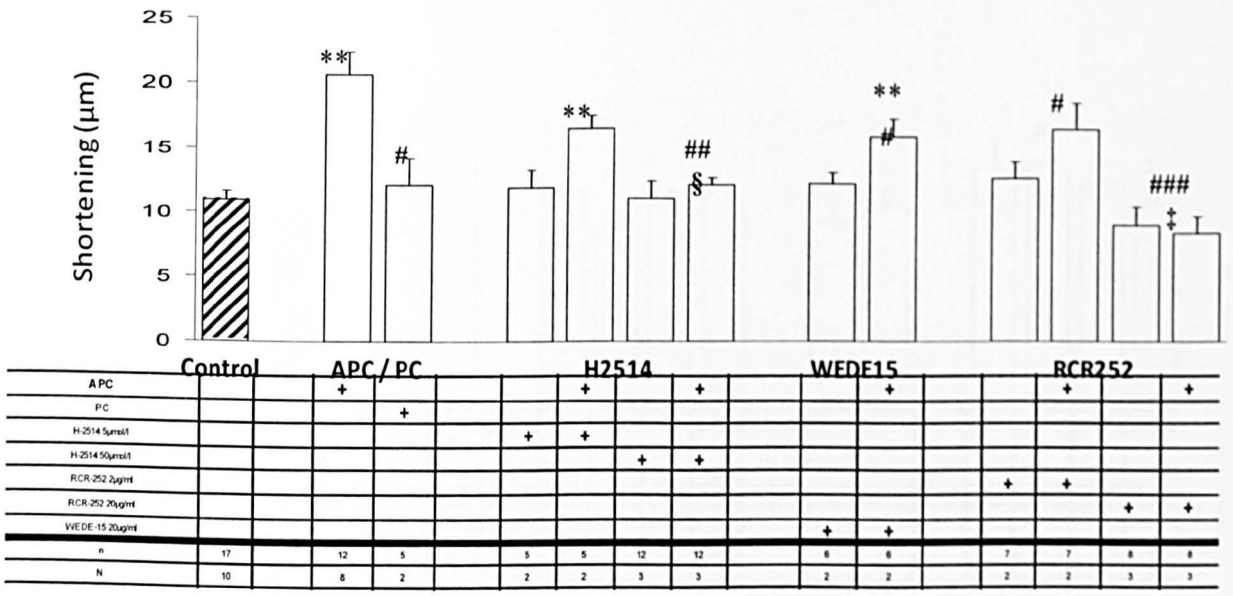


Figure 4.26 Histogram to show the effect of EPCR/PAR-1 blockade on APC induced shortening

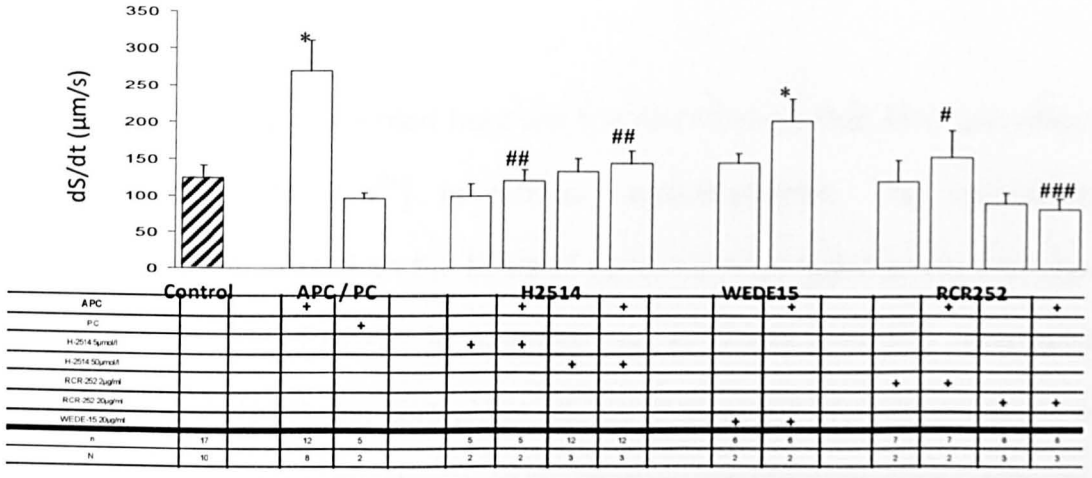


Figure 4.27 Histogram to show the effect of EPCR/PAR-1 blockade on APC induced maximum velocity (dS/dt)

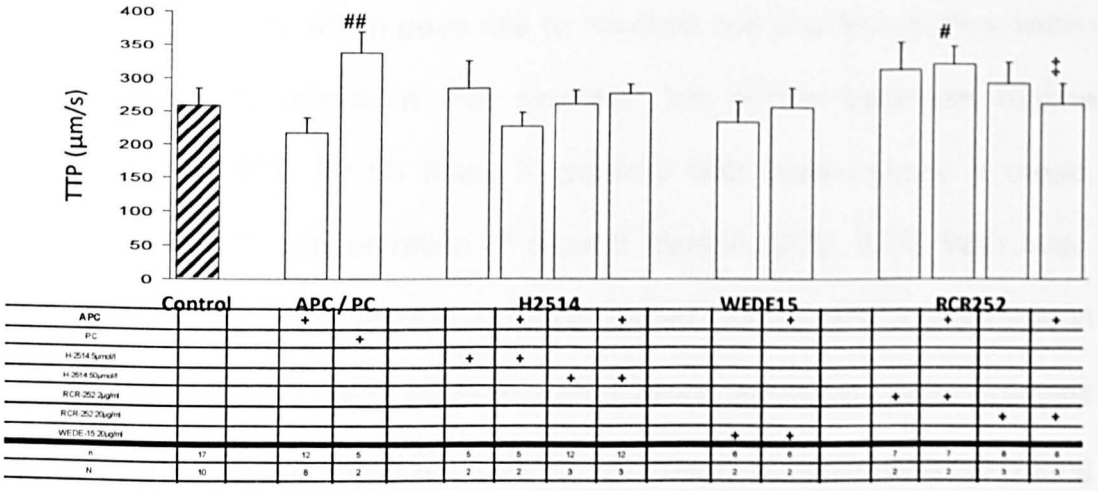


Figure 4.28 Histogram to show the effect of EPCR/PAR-1 blockade on APC induced time to peak (TTP)

Figure 4.26-8 Data are expressed as mean±SEM. *($p<0.05$) and **($p<0.01$) for mean test values versus controls. #($p<0.05$), ##($p<0.01$) and ###($p<0.001$) for mean test values versus 5nmol/L APC. §($p<0.05$) for 5µmol/L H2514 versus 50µmol/L H2514, ‡($p<0.05$) for 2µg/ml RCR252 versus 20µg/ml RCR252.

4.3 Discussion

To our knowledge, the data presented here are the first to show that APC can affect developed contraction and $[Ca^{2+}]_i$ in normal cardiomyocytes. The contractile response could be characterized on the basis of concentration-dependence and was mediated by EPCR and PAR-1. In contrast to APC-EPCR-PAR-1 mediated responses in other cells [271], the contractile effects peaked within a narrow and low range of APC concentrations. The lower concentrations tested were therapeutically relevant and gave rise to an increase in peak cell shortening without modification of $[Ca^{2+}]_i$. This suggested an effect of APC on myofilament sensitivity to Ca^{2+}. At higher concentrations, APC significantly altered calcium handling.

Interestingly, concentrations which gave rise to maximal cell shortening were within the range of therapeutic relevance. For example, the clinical treatment regime infusing 24ug/kg/hr of APC for 96 hours in patients with sepsis yields a mean, steady-state plasma APC concentration of around 1nmol/L [272, 273]. With inter-species differences reported to require a 10-fold higher human APC concentration for an equivalent effect on rodents [274], the peak response in rat cardiomyocytes shortening at around 5nmol/L would translate to ~0.5nmol/L in septic patients during APC treatment. Optimal dosing required to achieve organ-specific protection has never been evaluated. As APC concentration was increased the beneficial effect on contraction was lost, and the model predicted an eventual negative inotropic effect. Of relevance is the clinical finding that it is patients with the most severe sepsis and the most depressed protein C (PC) levels that derive most benefit from treatment [229, 275]. By contrast, relatively less sick patients do not derive benefit from APC

treatment [125], and where PC levels are near normal APC treatment could overshoot this window of positive effect in terms of cardiac contractility.

Of the strategies proven to be effective in sepsis, none specifically address cardiac morbidity. Clinical sepsis can lead to depressed myocardial function and mechanical dysfunction of isolated myocytes has been demonstrated in experimental models of sepsis as reduced cell shortening, reduced velocity of shortening and prolonged relaxation [276]. Furthermore sepsis-induced alterations in Ca^{2+} sensitivity of cardiac myofilaments have also been shown, and proposed to account for the reduced contractile function [277]. Our results suggest a mechanism at the single cell level to support such studies that suggest APC affects intrinsic heart contractility in sepsis, which has never been investigated experimentally.

The biphasic response we observe following APC exposure is of particular mechanistic interest. At lower concentrations (≤ 5nmol/L), an increase in contractility without significant modification of Ca^{2+} handling suggests an increase in myofilament sensitivity to Ca^{2+}. G protein coupled receptor mechanisms regulating changes in myofilament sensitivity to Ca^{2+} remain controversial [278]. However, GPCR activation and downstream protein kinase signalling has been reported in the failing human heart and in animal models [279, 280]. Knowledge of how APC signals via PAR-1 following binding to EPCR has arisen mainly through work on endothelial cells[271]. Pathways include MAPK phosphorylation [271], NFKb suppression and sphingosphine-1 [68]. What is already known regarding PAR-1 signalling networks could account for the pattern of effect on Ca^{2+} responsiveness seen in this study. PAR-1 can activate G-proteins to trigger a multitude of pathways and induce serine/threonine kinase signalling cascades in a concentration-dependent manner

[281]. Cleavage of Gq can lead to the activation of different isoforms of protein kinase C (PKC) [282, 283]. The subsequent phosphorylation of myofilament proteins [284] can have a variable effect on modulating Ca^{2+} sensitivity depending on the isoform sensitivities [285]. For example, the atypical PKC isoform, PKC-ζ, is responsible principally for an increase in myofilament sensitivity to Ca^{2+} due to dual phosphorylation [286] of cardiac troponin I (cTnI) and cardiac troponin T (cTnT) [287, 288]. Work using a PAR-1 agonist has shown that down-regulation of adenylyl-cyclase [281] could also indirectly increase Ca^{2+} responsiveness by reducing the effects of PKA on cTnI [289, 290]. Such pathways may account for the increase in peak cell shortening, without significant modification $[Ca^{2+}]_i$, at lower APC concentrations. Phosphorylation of cTnT [288, 291] and cTnI [286, 288] by PKC-α has been shown to reduce myofilament sensitivity to Ca2+ [288, 291], which could explain the lesser effect on contractility seen at APC concentrations > 5nmol/L. Furthermore PKC-β and PKC-δ can also reduce Ca^{2+} sensitivity via the PAR-1-Gq/PLC pathway [292]. In humans potential target sites for PKC have been identified on cTnI, leading to depressed contractility by destabilising the cTnI- cTnC complex [293]. It is likely that the end effects on Ca^{2+} responsiveness are mediated by a balance of the PAR activated pathways described, in a concentration and time-dependent fashion.

Of particular interest is the observation of APC-induced increases in both peak and resting $[Ca^{2+}]_i$ without the expected positive effect on cardiomyocyte contractility. Following an increase in myofilament responsiveness to $[Ca^{2+}]_i$ for lower concentrations of APC, we postulate a second pathway limiting this effect which would appear to persist as [APC] is increased. An increase of Ca^{2+} release from the sarcoplasmic reticulum (SR), through the ryanodine receptors (RyR2) and inositol 3-

phosphate receptors (IP3R) could explain the increase in $[Ca^{2+}]_i$ observed. A further mechanism which could account for the APC induced increase in Ca^{2+} relates to the dependence of Ca^{2+} handling upon the SR Ca^{2+} pump (SERCA2), and to a lesser extent the Na^{+}-Ca^{2+} exchanger (NCX). PKC-α increases phospholamban binding to SERCA2, thus reducing Ca^{2+} reuptake which would be consistent with the effect we see on resting $[Ca^{2+}]_i$. Li *et al* showed that phosphorylation of the Na^{+}-H^{+} exchanger by PKCε and PKCα impacts upon the function of NCX either inducing (ε), or preventing (α), the internal accumulation of Ca^{2+}[293]. At higher concentrations, APC may mimic a similar effect leading to an elevation in resting $[Ca^{2+}]_i$ as observed in this study. It is likely that increased diastolic $[Ca^{2+}]_i$ contributes to reduced myofilament responsiveness as the cell becomes saturated with calcium.

The concentration dependent effects we describe emphasise the likelihood of a balance of different mechanisms regulating Ca^{2+} homeostasis. With reference to our findings, Ca^{2+} sensitizing compounds have previously evaluated as therapeutic agents. By increasing contractility without affecting cellular Ca^{2+}concentration, the disadvantages of Ca^{2+} overload and increased energy requirements are avoided[294]. Our results suggest that APC may mimic other inotropic drugs such as digoxin that exhibit a therapeutic window for efficacy. As such these findings, with a basis in sepsis could have relevance to the therapeutics of myocardial depression in general. Escalation dosing to achieve optimal cardiovascular recovery may be useful. Moreover, the development of non-anti-coagulant APC variants, which retain EPCR-PAR-1 signalling but minimise bleeding risk [274, 295] may further encourage such an approach.

In conclusion, in this chapter we show that APC has a positive inotropic effect on cardiomyocytes, specifically modulating shortening by a mechanism resulting in a bell shaped curve of effect with a maximum at 5nM APC. Further, APC induces an increase in $[Ca^{2+}]_i$ in a dose dependent manner with a maximum response reached at 100nM. The presence of EPCR and PAR-1 on cardiomyocytes is confirmed by QPCR, Western blotting and immunohistochemistry, and blockade of these receptors significantly reduced the effects of APC induced shortening. The data suggest that APC could have beneficial effects on cardiovascular function resolution in sepsis through mechanisms mediated through the EPCR-PAR-1 axis.

Chapter 5 The Effect of Thrombin alone or with APC on Cardiomyocytes

5.1 Introduction

Having demonstrated that APC has a positive inotropic effect on cardiomyocytes which appears to be PAR-1 dependent, this chapter specifically examines the exclusive effect of thrombin on shortening and whether this occurs through PAR-1. The shortening effect of APC is then tested in the presence of thrombin.

There is limited data on thrombin's cellular actions in the adult heart. Thrombin increases spontaneous automaticity, elevates $[Ca^{2+}]_i$ in a range of cardiomyocyte preparations [240, 296, 297] and induces pro-arrhythmic events during early reperfusion in intact adult hearts [238, 298].

Cellular responses to thrombin are mediated by the family of G-protein–coupled protease-activated receptors (PARs), of which PAR-1 is the prototype. Additional PAR family members have been identified. PAR-2 has 30% sequence identity to PAR-1 and is detected by Northern blot analysis in several tissues, including (at low levels) the heart [299]. PAR-3 and PAR-4 are more recently identified PAR family members. PAR-3 and PAR-4 have been studied exclusively in the context of platelet aggregation; potential functions in other tissues remain mostly unexplored[300]. PAR-2 is activated by limited proteolysis of its amino-terminal exodomain by trypsin (but not thrombin) or by SLIGRL, the PAR-2 tethered-ligand sequence [283].

PAR-1 activation has been linked to a diacyl-glycerol/ protein kinase C pathway that activates Na^+-H^+ exchange and increases contractile performance in rat adult cardiomyocytes [301] however study of the functional significance of PAR-1 is incomplete. Although there is a relative abundance of data characterising the

phenotype of thrombin at the endothelial surface, there is a paucity of data detailing its cellular actions on the heart. Furthermore many studies look at neonatal rat ventricular myocytes versus adult cardiomyocytes. Recent studies suggest that PAR-1, PAR-2 and PAR-4 are expressed by cardiomyocytes, however their distribution and functional roles across species can vary [282].

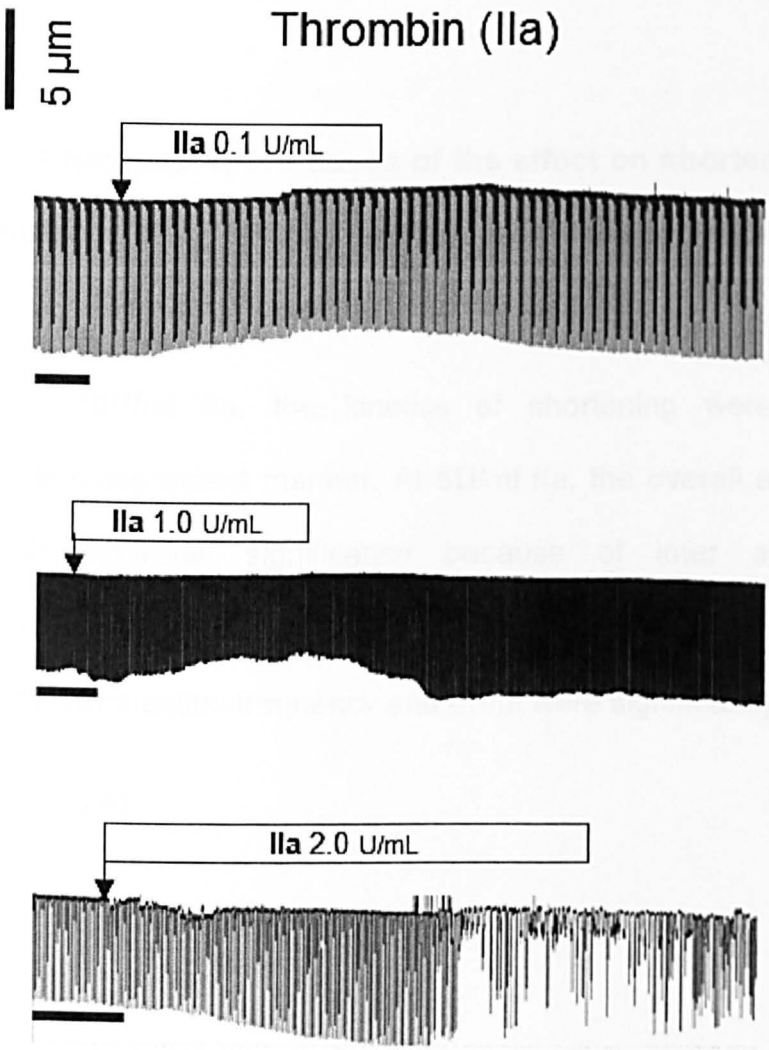
PAR-1 has historically been recognised as the prototypical receptor for thrombin, however with increasing recognition of the pleiotropic nature of PAR-1 mediated APC signalling, there is much debate about how both agonists can act mutually exclusively. The relatively long half-life of APC, at approximately 20 minutes [42] maintains a circulating concentration of 38pmol/l [19]. This contrasts with the much shorter half-life of thrombin and is likely to relate to the importance of maintaining vascular patency following localization of clot formation. Thrombin needs to be rapidly inactivated in solution to ensure that its potent effects are not further disseminated. *In vitro* studies would suggest that APC is 10^3 to 10^4 fold less potent than thrombin in cleaving PAR-1 [249] and therefore how the APC effect might coexist *in vivo* when thrombin generation is in excess is the focus of much research (see introduction).

In this chapter we study the effect of thrombin on shortening, the effect of thrombin with APC and the role of PAR-1.

5.2 Results

5.2.1 The Effect of Thrombin (IIa) on Cardiomyocyte Shortening

As IIa concentration was increased >1U/ml, cardiomyocytes became less responsive to electrical stimulation and fibrillation became evident. In certain cases, these effects could be alleviated by limiting duration of IIa application. No significant increase in shortening was observed at any IIa concentration. However, IIa concentrations of 1-10U/ml caused negative effects on cardiomyocyte function ((Figure 5.1).



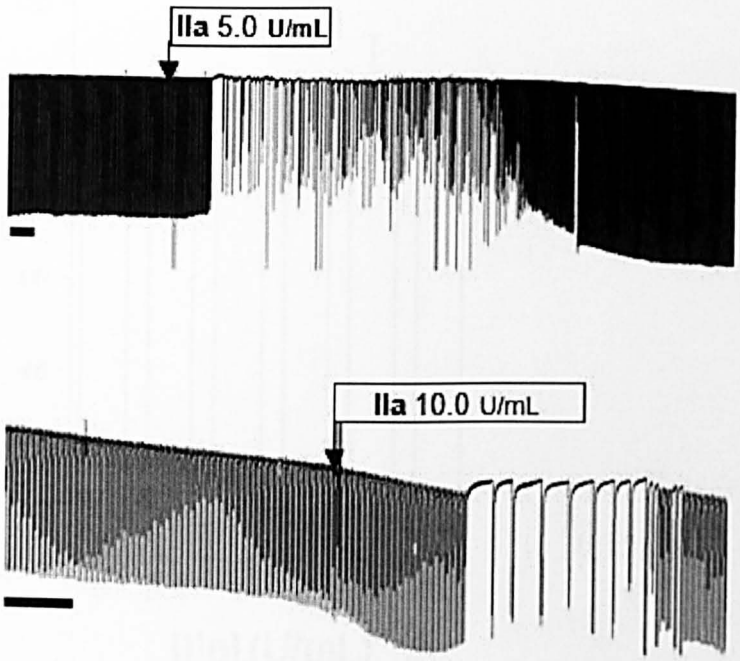


Figure 5.1 Representative traces of the effect on shortening for increasing IIa concentrations (0.1-10U/ml); IIa application and concentration indicated by arrow above trace) Black bar represents timescale of 1 min.

Between 1-10U/ml IIa, the kinetics of shortening were also modified in a concentration-dependent manner. At 5U/ml IIa, the overall effect on shortening did not reach statistical significance because of inter and intra-variability of cardiomyocyte shortening amplitude with respect to the lack of response to electrical stimulation. Contraction frequency and dS/dt were significantly reduced.

(Figures 5.2-5.4).

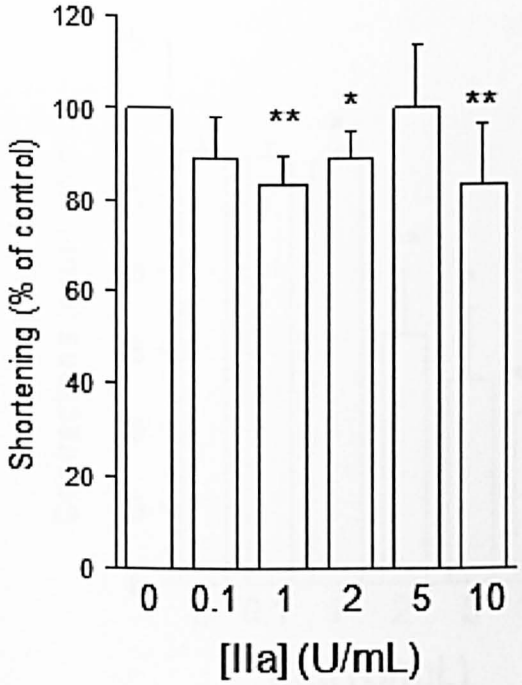
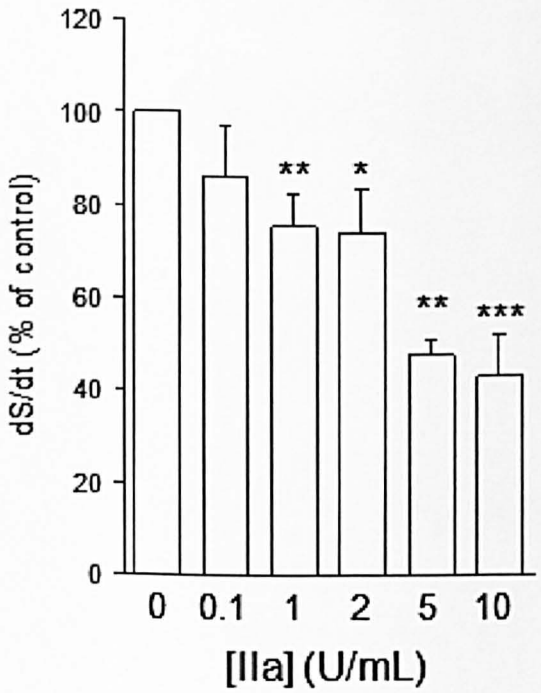


Figure 5.2 Histogram to show the mean amplitude of shortening in response to increasing IIa concentrations (above) relative to control (* p<0.05)

Figure 5.3 Histogram to show the maximum velocity of shortening in response to increasing IIa concentrations (below)



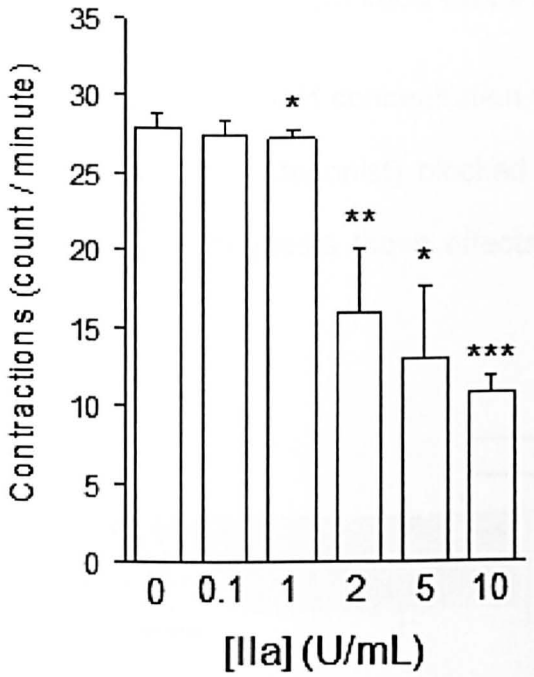


Figure 5.4 Histogram to show the contraction frequency in response to increasing IIa concentrations

5.2.2 The Effect of PAR-1 blockade on IIa induced cardiomyocyte responses

Using 2U/ml as the threshold concentration for the toxic effects of IIa, pre-incubation with WEDE15 (PAR-1 antagonist) blocked the IIa effect on contraction frequency and relaxation. This suggests these effects are therefore PAR-1-mediated (Figure 5.6, Table 5.1).

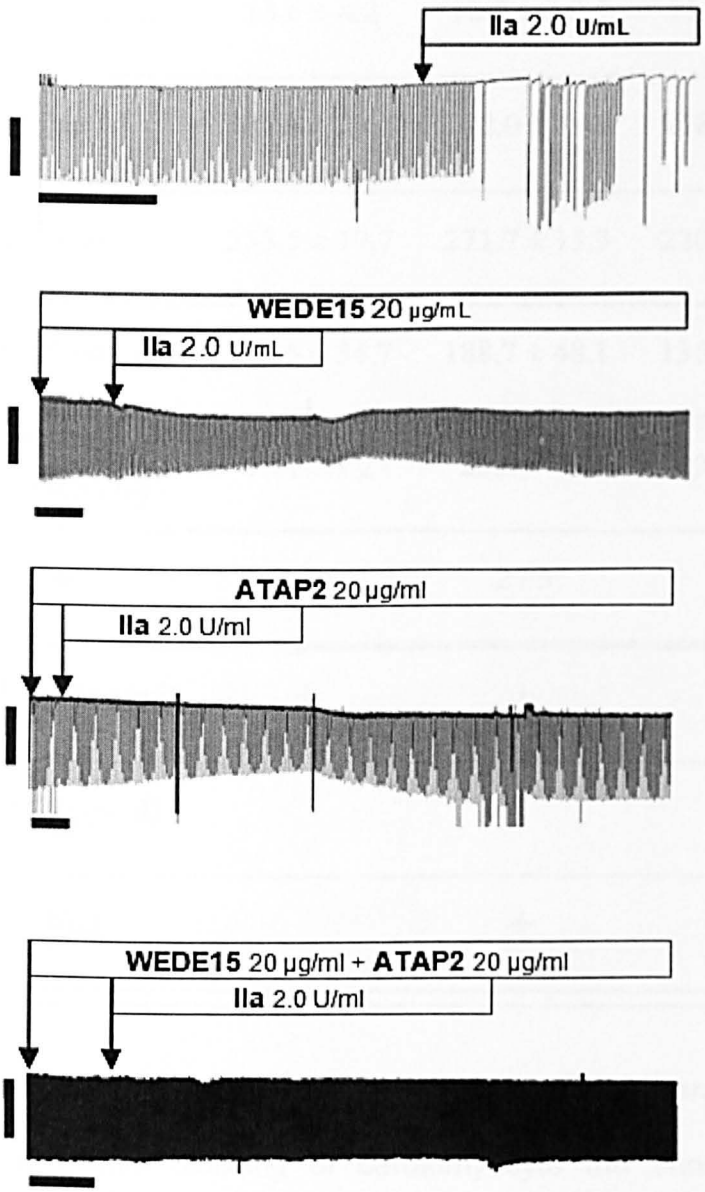


Figure 5.5 Representative traces to show the effect of PAR-1 blockade on IIa (2U/ml) induced shortening. Application of IIa indicated by arrow below. IIa applied alone as control (top trace). Pre-incubation with specific blocking agent to PAR-1 20 µg/ml WEDE15 or 20 µg/ml ATAP2, or both together, indicated above the trace

Shortening (µm)	13.6 ± 4.2	10.8 ± 3.9 ‡	11.3 ± 2.24	9.12 ± 2.23 *
dS/dt (µm/s)	137.8 ± 29.2	112.0 ± 19.9	118.7 ± 50.4	98.9 ± 22.2
TTP (ms)	253.5 ± 17.7	271.7 ± 15.9	220.4 ± 47.6	254.0 ± 66.6
T_{half} relaxation (ms)	168.6 ± 34.7	188.7 ± 48.1	136.3 ± 37.7	172.3 ± 49.8
Contractions (count / minute)	27.4 ± 1.2	26.0 ± 0.7	29.2 ± 1.3	25.7 ± 2.5
N / n	2 / 5	2 / 5	2/5	2/5
WEDE15 (20 µg/ml)	+	+		
ATAP2 (20µg/ml)			+	+
IIa 2U/ml		+		+

Table 5.1 Table to show the effect of PAR-1 blockade on IIa induced shortening parameters. Partial blocking of cardiomyocyte the shortening response to IIa (2U/ml) was observed following pre-incubation with WEDE15 or ATAP2. ‡ for $p<0.05$ between WEDE15 and WEDE15 plus IIa. * for $p<0.05$ between ATAP2 and ATAP2 plus IIa.

5.2.3 The Effect of APC on Cardiomyocyte Shortening in the Presence of IIa

Given that PAR-1 activation on cardiomyocytes resulted in different responses depending on the agonist, studies were performed with APC and IIa in combination. APC (5nmol/L) was applied with IIa (0.1 or 1.0U/ml) in 3 settings: APC before, simultaneously or after IIa (Figure 5.7). APC was not tested with thrombin concentrations ≥2U/ml as fibrillation does not represent a physiologically functional state.

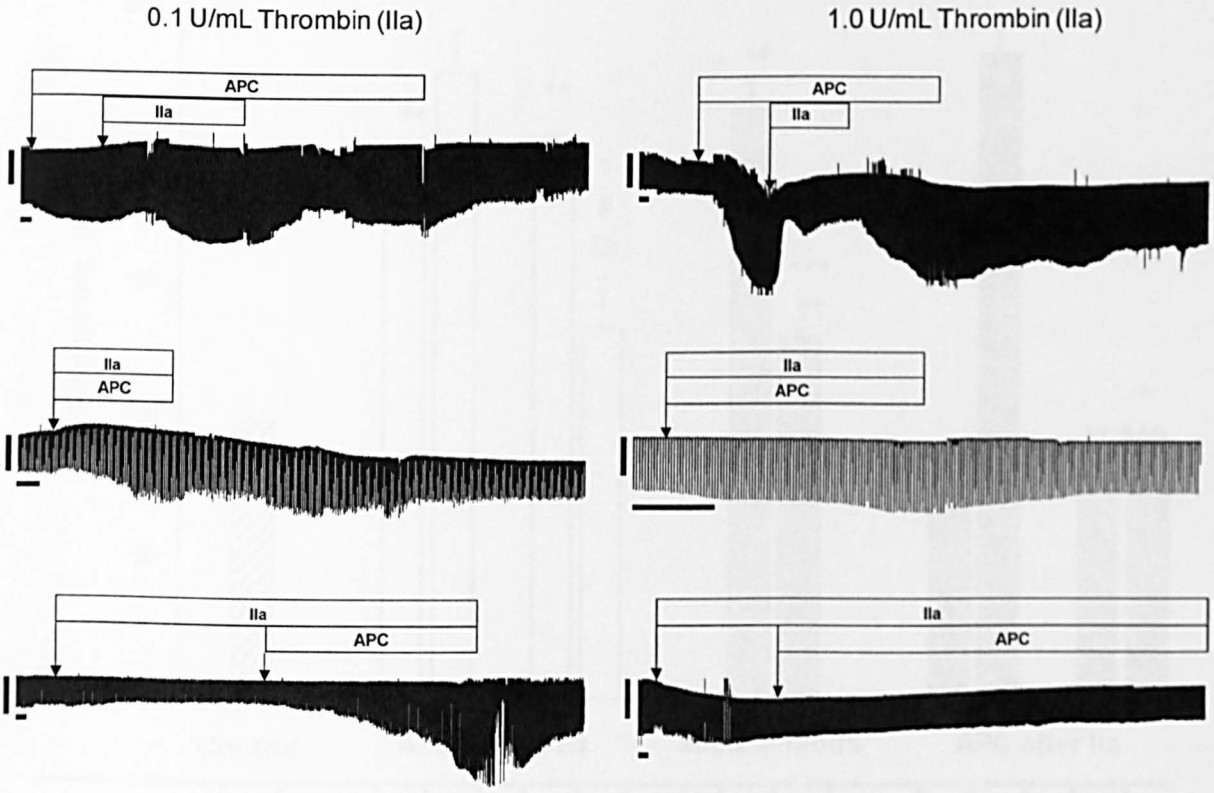
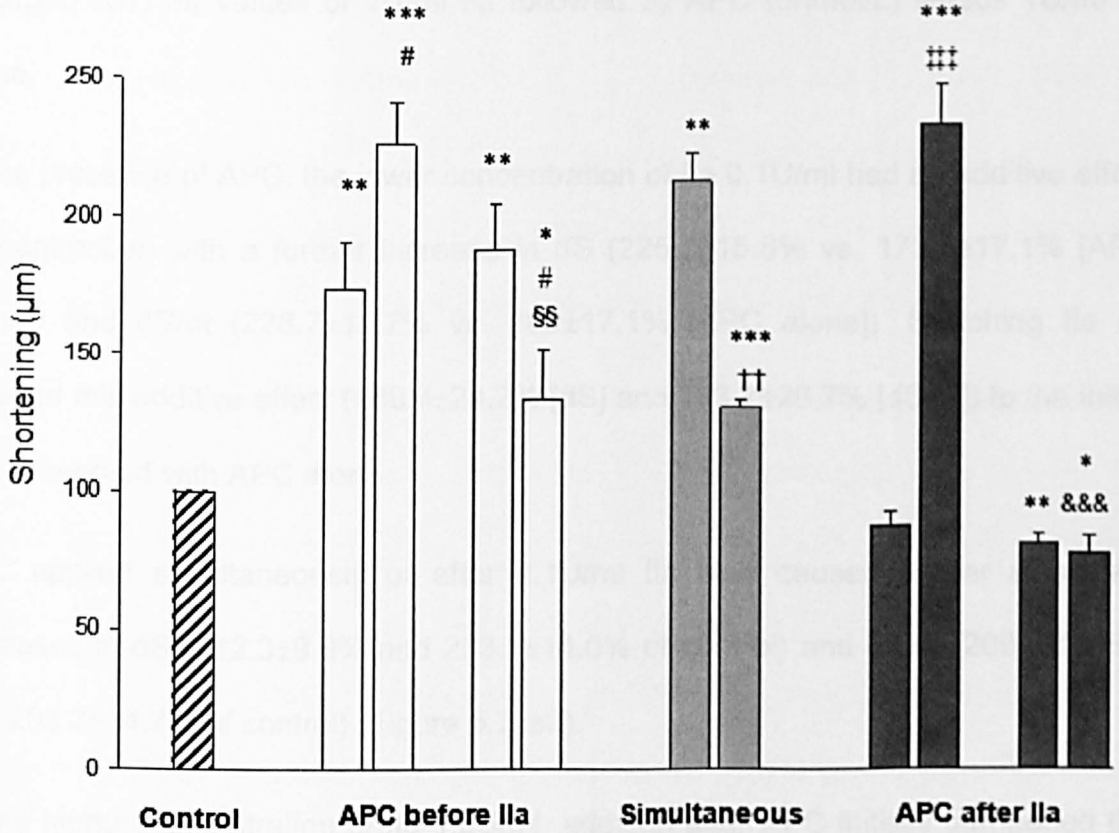


Figure 5.6 Representative traces to show changes in APC induced shortening in the presence of IIa. Cardiomyocytes were placed in the perfusion a chamber and shortening recorded in response to different combinations of APC and IIa as shown.

Order and duration of perfusions of APC and IIa through the flow chamber for each condition are indicated in open boxes above each trace. The horizontal black bar below each trace represents one minute and the vertical black bar on left represents 5µm. Lower IIa concentration (0.1 U/ml) is applied after (*upper trace*), simultaneously (*middle trace*) and before (*lower trace*) 5 nmol/L APC. Higher IIa concentration (1 U/ml) is applied after (*upper trace*), simultaneously (*middle trace*) and before (*lower trace*) 5 nmol/L APC.



APC 5 nM	-		+	+		+	+		+	+		-	+		-	+
IIa 0.1 U/mL	-		-	+		-	-		+	-		+	+		-	-
IIa 1 U/mL	-		-	-		-	+		-	+		-	-		+	+
n	33		7	7		6	6		7	7		5	5		5	5
N	16		4	4		3	3		2	3		2	2		2	2

Figure 5.7 Histogram to show changes in APC induced shortening in the presence of IIa; mean ds/dt in response to APC with IIa (expressed as percentage of control). Data expressed as mean±SEM. *($p<0.05$), **($p<0.01$) and ***($p<0.001$) for mean test values versus control, #($p<0.05$) for values of APC followed by 0.1U/ml IIa versus 5nmol/L APC alone, §($p<0.05$) and §§($p<0.01$) for values of APC followed by 1 U/ml IIa versus 5nmol/L APC alone, †($p<0.05$) and ††($p<0.01$) for simultaneous APC and 0.1U/ml IIa versus simultaneous APC and 1U/ml IIa, ‡‡‡($p<0.001$) for values of 0.1U/ml IIa followed by APC (5nmol/L) versus 0.1U/ml IIa alone, &&&($p<0.001$) for values of 1U/ml IIa followed by APC (5nmol/L) versus 1U/ml IIa alone.

In the presence of APC, the lower concentration of IIa 0.1U/ml had an additive effect on contraction with a further increase in dS (225.7±15.6% vs. 173.3±17.1% [APC alone]) and dS/dt (228.7±18.7% vs. 189±17.1% [APC alone]). Switching IIa off reverted this additive effect (188.4±24.2% [dS] and 183.7±26.7% [dS/dt]) to the initial level observed with APC alone.

APC applied simultaneously or after 0.1U/ml IIa also caused similar significant increases in dS (212.3±9.9% and 233.1±14.0% of control) and dS/dt (209.6±21.5% and 203.3±14.7% of control) (Figure 5.7 left).

At the higher concentration of IIa 1.0U/ml, addition after APC initially dampened the dS increase but overall, there was a significant increase relative to control (133.7±17.9% vs. 187.9±16.7% [APC alone]). With simultaneous APC and 1U/ml IIa, the significant dS increase (129.8±3.4%) was less compared to 0.1U/ml IIa. No significant dS effect occurred when APC was applied after 1.0U/ml IIa (77.5±6.3%

vs. 81.1±4.2% [IIa alone]) (Figure 5.7 right). A summary of the changes is given in Figure 5.7.

Analysing the kinetics of shortening, similarly, dS/dt increased only when APC was applied before or simultaneously (142.4±22.1% and 135.7±3.6% of control) with IIa (data not shown).

5.3 Discussion

With limited literature on the cellular actions of thrombin on adult ventricular myocytes, and no data defining shortening responses, our results reveal a concentration-dependent negative effect on cardiomyocyte function. From a threshold concentration of 1 U/ml thrombin, irregular and ineffective contractions became more evident. Relating this to processes *in vivo*, thrombin concentrations found in vascular areas of clotting/thrombosis are in the range 1-10U/ml [302].

Jiang *et al* proposed that elevations of $[Ca^{2+}]_i$ in adult rat cardiomyocytes were PAR linked on the basis of the identification of PAR-1 mRNA and responses to SFLLRN [240] PAR-1 has been implicated in the actions of thrombin. However, SFLLRN typically elicits biochemical responses that are more robust than those elicited by thrombin. This could result from the actions of SFLLRN at both PAR-1 and PAR-2, because SFLLRN activates both, whereas thrombin is selective for PAR-1[303]. Thrombin 1U/ml and SFLLRN induce rapid and transient increases in inositol triphosphate (IP_3) and inositol bisphosphate (IP_2) in cardiomyoytes, which are followed by a more sustained accumulation of inositol monophosphate (IP_1).

For both agonists, the increase in calcium was dose dependent (maximum at 300 mmol/L) and was completely reversed during 3 to 4 minutes of washout. PAR-1 and

PAR-2 both couple to increases in spontaneous automaticity and elevations in calcium. These responses would tend to predispose to arrhythmias. In the presence of ATAP2 or WEDE15, the fibrillation and pauses seen with 2U/ml thrombin were abrogated supporting a PAR-1-mediated pathway. The reduction in shortening however was only blocked by conjugated effect of ATAP2 plus WEDE15, raising the possibility of more than one mechanistic pathway to explain the shortening response to thrombin [304]. Co-expression of multiple thrombin sensitive PARs, including specific intracellular effector pathways may explain the degree of response to a range of thrombin concentrations [300]. Further work is required to dissect the nature and potential effects of these interactions.

Given that thrombin appears to cleave the same scissile bond on PAR-1 with much higher efficiency than APC, the significance of APC signalling in the presence of thrombin has been questioned [305]. Our findings that the positive inotropic effect of APC alone is detectable in the presence of thrombin at 0.1 and 1.0U/ml, (equivalent to ~0.7nmol/L and 7nmol/L respectively) are therefore important considerations to this debate. Furthermore, it substantiates the findings in a model of endothelial cell permeability whereby APC could enhance barrier integrity despite the presence of up to 1.56nmol/L thrombin. Schuepbach *et al* perform clevage studies showing that APC can mediate significant additional cleavage of PAR-1 in the presence of up to 360pmol/L thrombin [76]. Thrombin (50pmol/L) alone has been shown to induce barrier protection [268] later becoming barrier-disruptive at higher doses (50nmol/L) [306]. Recent work extends this PAR-1 dependent paradoxical role of thrombin for other anti-inflammatory actions on endothelial calls up to a concentration of 100pmol/L [245]. Using our cardiomyocyte model, we show that at 100pmol/L there is no significant negative effect of thrombin moreover a beneficial effect in the

presence of APC. Irrespective of the sequence in cardiomyocyte exposure to APC and thrombin (0.1U/ml), a further increase in contraction was observed. This was also true for thrombin (1U/mL) where the negative inotropic effect was reversed when applied simultaneously or after APC. Given our speculation that APC ≤5nmol/L alone may increase myofilament sensitivity to $[Ca^{2+}]_i$, this additive effect could be explained by coupling of thrombin to downstream pathways. These include PLC, ERK and p38, which have been shown to alter calcium metabolism [300] and thus optimise interactions between calcium and the contractile apparatus. Recent findings of downstream targets from vascular PAR-1 have been proposed to explain the signalling pathway selectivity by thrombin and APC. The PAR-1-sphingosine-1-phosphate 3 (S1P3) pathway has been linked to a worse outcome in sepsis through vascular leakage and endothelial-barrier disruption. Conversely, PAR-1 cross-activates S1P1 and S1P1 agonism can protect against detrimental effects from high levels of thrombin [78]. This is likely to be affected by the availability of EPCR to PAR-1 at the cell surface but little is known on their relative expression ratios in determining differential responses between thrombin and APC. More recently it has been suggested that cavelolae have a central role in protease selective PAR-1 signalling in endothelial cells [307] however this is less well defined for cardiomyocytes. Here we address opposing roles for PAR-1 on cardiomyocytes and support the concept that PAR-1 dependent signalling may contribute to the cardioprotective effects of APC in sepsis. The physiological relevance of the EPCR–APC–PAR-1 axis in sepsis may be related to topographical dynamics, both at the vascular site-specific level and at the level of the single cell. Firstly, the local *in vivo* environment of the vascular bed is difficult to reproduce experimentally. Although thrombin concentrations are initially much greater at sites of injury, removal by

circulating inhibitors and blood flow gradients may contribute to a situation where APC activity prevails to rescue cells from the progressive effects of sepsis [247].

Chapter 6 The Effect of a Protein Kinase C alpha (PKCα) Inhibition on APC or IIa on CM Shortening

6.1 Introduction

In the previous chapter, we demonstrate contrasting effects of thrombin and APC on cardiomyocytes, which appear to be mediated through the same receptor PAR-1. Furthermore, the effects on shortening by the less potent PAR-1 agonist, APC (5nM) can be seen in the presence of thrombin. As with endothelial barrier protection [78], downstream signalling targets may provide insight about the signalling pathway selectivity of thrombin/APC for PAR-1. Although 4 types of PARs have been identified, their discrete physiological and pathological roles are still being unravelled. A summary of the signal transduction pathways involving PARs 1, 2 and 4 in cardiomyocytes is illustrated below [282].

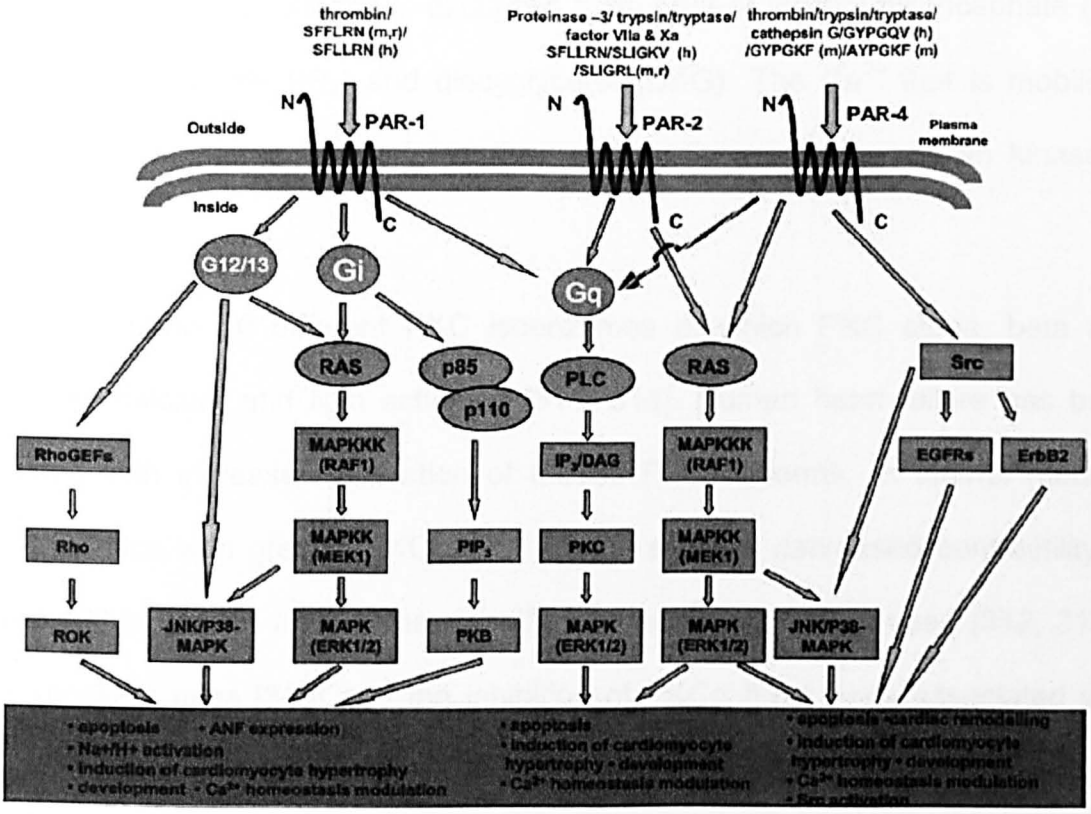


Fig. 2. Signal transduction pathways involving protease activated receptors 1, 2 and 4 in cardiomyocytes. Abbreviations used: DAG, diacylglycerol; EGFR, epithelial growth factor receptor; ERK, extracellular signal-regulated protein kinase; G_q, G_i, and $G_{12/13}$, G protein family members; MAPK, mitogen-activated protein kinase; MAPKK/MAPKKK p38-MAPK, p38-mitogen-activated protein kinase; MEK, mitogen-activated extracellular signal regulated kinase kinase; PIP_3, phosphatidylinositol 1,4,5-trisphosphate; PIP_2, phosphotidylinositol 4, 5-biphosphate; PI, phosphotidylinositol; PKB, protein kinase B; PKC, protein kinase C; PLC, phospholipase C; src, src, proto-oncogene from rous sarcoma virus; JNK, c-Jun amino-terminal kinase MAPKK, MAPK kinase (MEK); MAPKKK, MAPKK kinase (RAF); Ras, transforming oncogene G protein $pp60^{src}$ and related kinases; RhoGEF, Rho G-protein nucleotide exchange factor. Some intermediates and precursors have been omitted for the sake of clarity. Although it is possible that PAR-2 and PAR-4 also signal through other G-proteins, data for this is presently unavailable.

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Figure 6.1 A summary of the signal transduction pathways involving PARs 1,2 and 4 in cardiomyocytes (Barnes et al, 2004) [282]

In the context of intracellular signalling, PAR-1 is the most studied PAR [308]. The coupling of PAR-1 to several G proteins suggests multiple signalling pathways for inducing the tyrosine kinase signalling cascade [309]. In rat ventricular cardiomyocytes, thrombin has been reported to activate the phosphoinositide hydrolysis and ERK pathway [238, 240, 283].

PKC functions downstream in the PAR-1 signal transduction pathway [310, 311] and has been reported to regulate contractility of cardiomyocytes [312, 313] Activation of

PAR-1 leads to phospholipase C catalysed hydrolysis of inositol bisphosphate (IP_2) to inositol triphosphate (IP_3) and diacyglycerol (DAG). The Ca^{2+} that is mobilised from intracellular stores is used together with DAG to activate protein kinase C (PKC) [309].

There are around 10 different PKC isoenzymes of which PKC alpha, beta and gamma are calcium and lipid activated [311, 314]. Human heart failure has been associated with increased activation of theses PKC isoforms. In animal models, transgenic mice with greater PKC alpha activity showed decreased contractility to suggest that increased PKC alpha signalling is detrimental to the heart [312, 313] . PKCα knockout mice [315] and the inhibition of PKCα have been associated with protection from heart failure [316].

The mechanism for how PKCα inhibition specifically attenuates heart failure is not fully understood. Possible explanations include phosphorylation of phospholamban (PLN), which in turn regulates SERCA function controlling Ca^{2+} load and transient. Alternatively, phosphorylation of myofilament proteins with augmented Ca^{2+} handling to enhance their function may be the dominant mechanism [316].

Using a PKC antagonist, Ruboxistaurin, Liu *et al* showed enhanced cardiac function in mice. Although the PKC inhibitors can demonstrate cross selectivity, the cardioprotective effect in this study was not seen in PKC alpha knockout mice suggesting mediation through PKCα [310].

In Chapter 4, we suggested that the positive inotropic effects of APC arise through alterations in myofilament sensitivity to calcium. As PKCα has been shown to reduce myofilament sensitivity to Ca^{2+}, we will now investigate its role as a downstream mediator of the PAR-1-mediated effects by APC and thrombin.

In this chapter, we look at the effect on shortening of using a PKCα inhibitor with 2 different concentrations of APC;

5nM APC as this induced the peak shortening effect, in the absence of a significant rise in $[Ca^{2+}]_i$

50nM APC as this induced a significant rise in $[Ca^{2+}]_i$, but not shortening

and, 1U/ml IIa known to increase $[Ca^{2+}]_i$, but did not increase shortening

Bearing in mind the calcium dependent negative effect of PKCα on contractility, we hypothesise that addition of a PKCα inhibitor could enhance the effect of 50nM APC and 1U/ml thrombin on cardiomyocyte shortening by increasing myofilament sensitivity.

6.2 Results

6.2.1 Regulation by PKCα of APC and IIa-induced effects on cardiomyocyte shortening

APC (5 or 50nM) was applied to contracting myocytes in a steady state. After inducing a shortening response the PKCαI Go 6976 (5nM) was applied in continuum with APC and the effect on shortening observed.

5nM APC + PKCαI;

Addition of Go 6976 produced little augmentation of cardiomyocyte shortening (+2.4±0.8% of control)

50nM APC + PKCαI;

Addition of Go 6976 produced a significant shortening effect (from 6.35±0.96μm in control to 11.56±0.78μm in the presence of 5 nmol/L Go 6976).

Then, IIa (1U/ml) was applied to contracting myocytes in a steady state. In the absence of any significant increase in shortening response, PKCαI Go 6976 (5nM) was applied in continuum with IIa and the effect on shortening observed.

1 U/ml IIa + PKCαI;

With IIa 1U/ml, which has a negative contractile effect on its own (-18.8±6.5%), addition of Go 6976 also increased shortening significantly (180.6±36.7% of control).

Inhibition of PKCα in control conditions without activation of PAR-1 did not significantly modify cardiomyocyte shortening or kinetics. This suggests that PAR-1 activation of PKCα is involved and critically influences the cardiomyocyte response to higher concentrations of APC or thrombin.

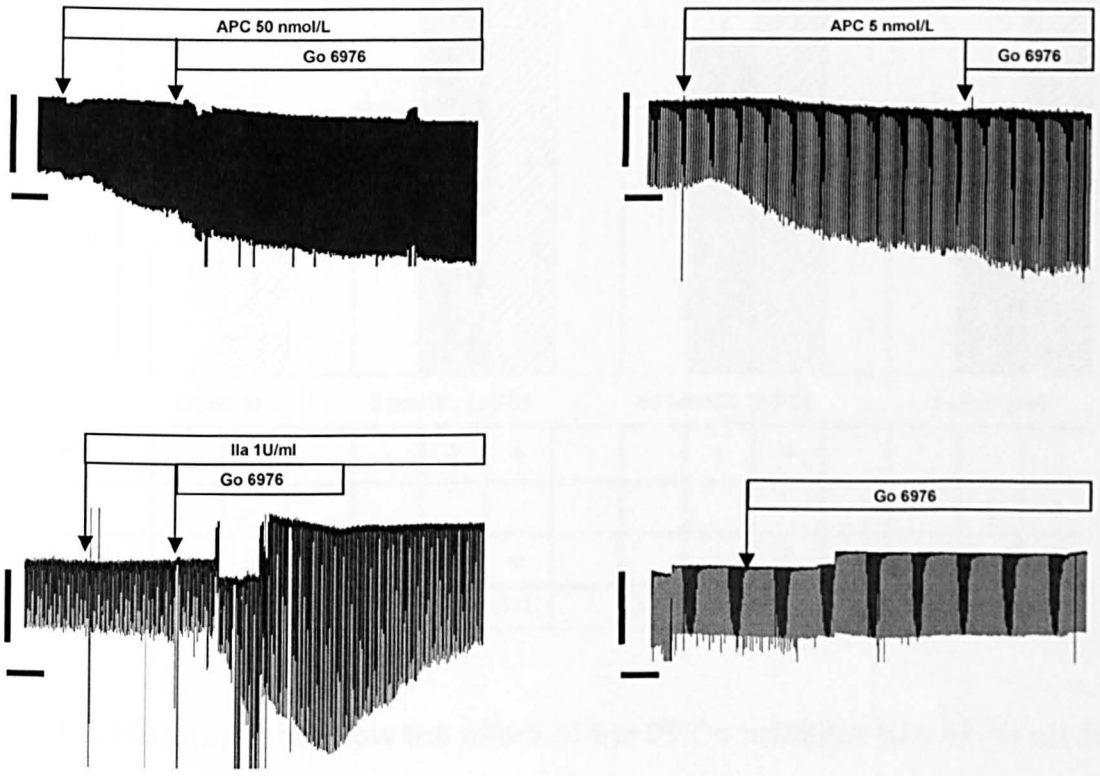

Figure 6.2 Representative traces to show the effect of a PKCα inhibitor Go 6976 on APC and IIa induced shortening

APC 5nM (top right) or APC 50nM (top left) applied before Go 6976 (top traces).

IIa 1U/ml (bottom left) applied before Go 6976. Go 6976 applied alone (bottom right). Point and duration of application of APC/IIa/Go 6976 shown above traces. (Go6976 5nM used in all experiments)

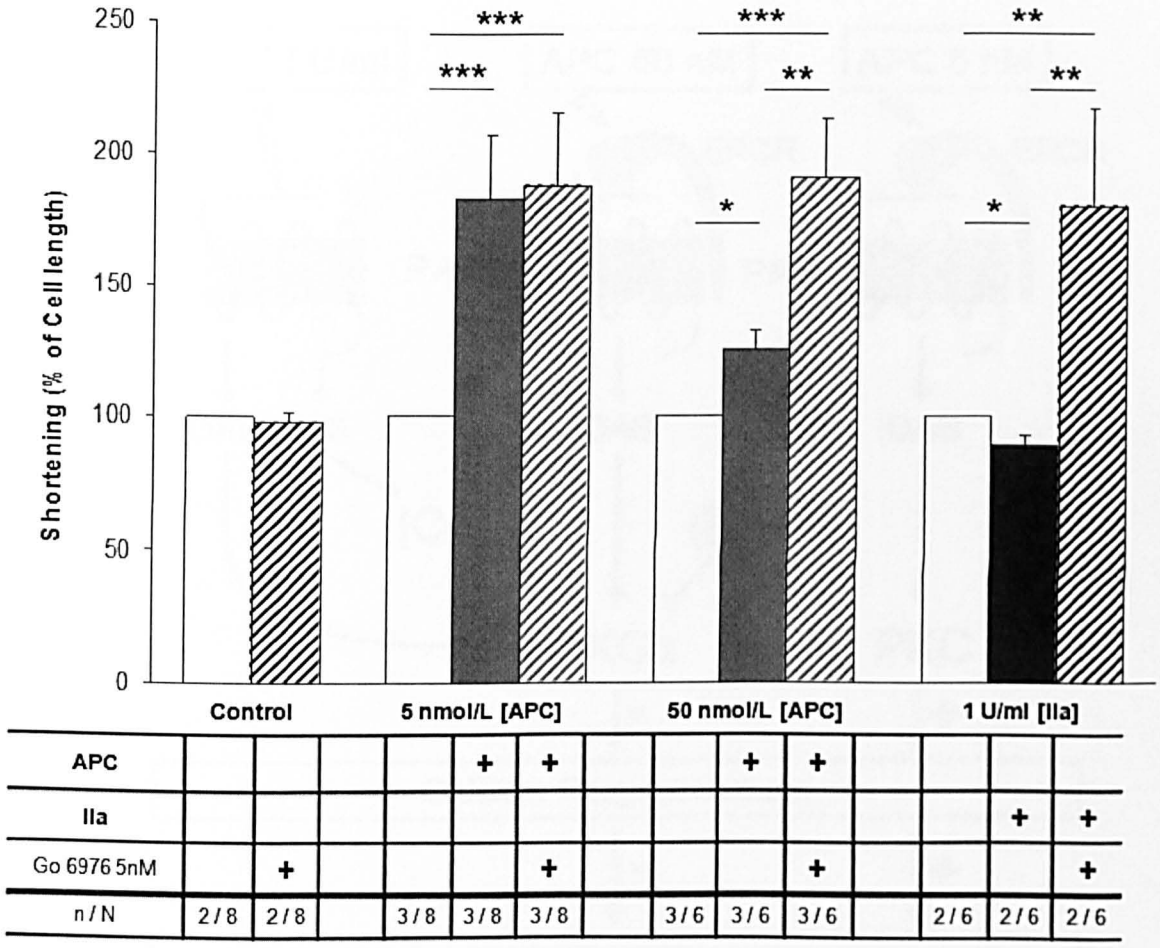

Figure 6.3 Histogram to show the effect of the PKCα inhibitor (Go 6976) on APC and IIa induced shortening (white bars control , diagonal striped bars-with Go 6976, light grey shaded bars- APC 5 and 50 nm, black shaded bars- IIa 1 u/ml, * $p< 0.05$, **$p<0.01$, ***$p<0.001$)).

6.2.2 Proposed pathway for APC and IIa Effects on CM shortening and $[Ca^{2+}]_i$

Based on the results from this chapter and evidence from the literature, we offer mechanistic insight in to the pathways that might account for the differential signalling outcomes when PAR-1 is activated by APC or IIa (Figure 6.4).

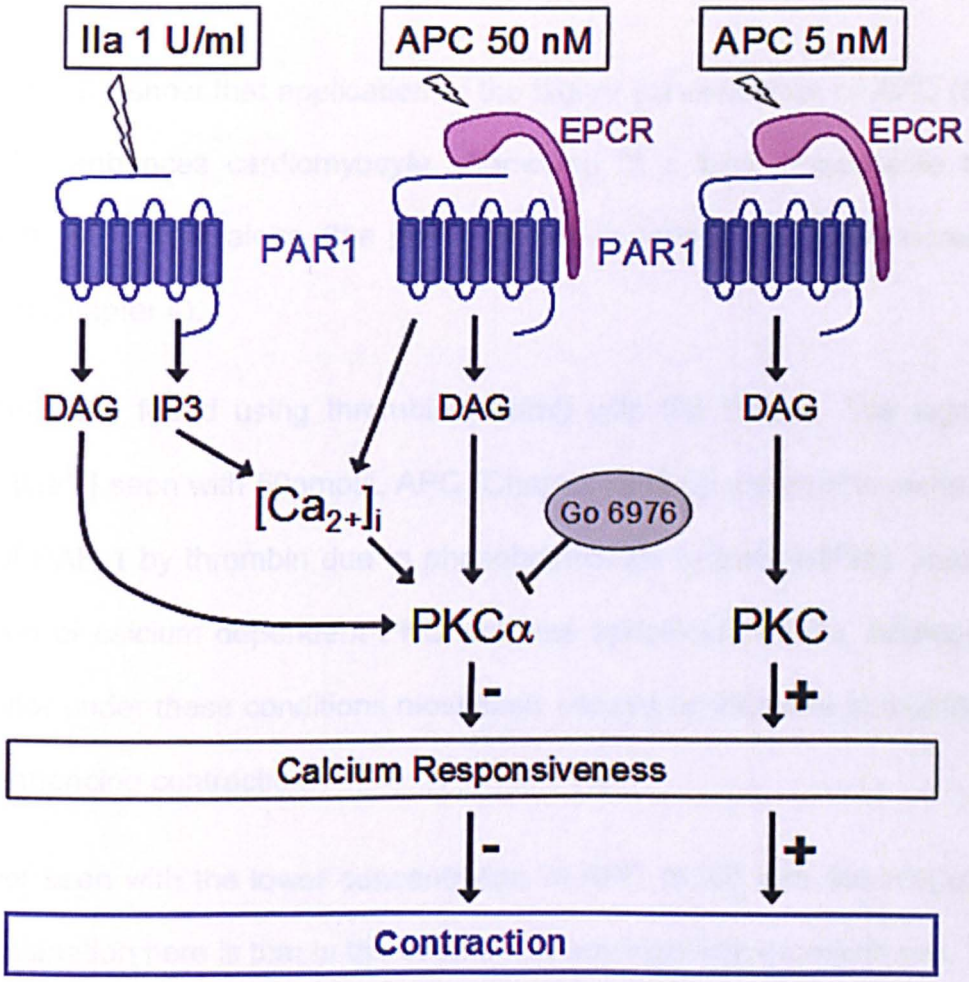

Figure 6.4 Schematic diagram proposing signalling pathways to account for the effects of APC and IIa on cardiomyocyte shortening and $[Ca^{2+}]_i$

Left pathway; IIa mediates an increase in $[Ca^{2+}]_i$ through phosphoinositide hydrolysis leading to activation of Ca^{2+} dependent PKCα which in turn reduces myofilament sensitivity to calcium and therefore contraction. APC at the higher concentration 50nM has as similar effect through PAR-1 whereas at the lower concentration 5nM there is no significant rise in $[Ca^{2+}]_i$, and in the absence of PKCα activation, myofilament sensitivity is increased leading to increased contraction as seen in experiments (Chapter 4).

6.3 Discussion

In this chapter, we show that application of the higher concentration of APC (50nM), with a PKCαI enhances cardiomyocyte shortening to a level comparable to that achieved with APC 5nM alone (the [APC] shown to induce the peak increase in shortening in Chapter 4).

Similar results are found using thrombin (1U/ml) with the PKCαI. The significant increase in $[Ca^{2+}]_i$ seen with 50nmol/L APC (Chapter 4) likely represents comparable activation of PAR-1 by thrombin due to phosphoinositide hydrolysis[238] leading to the activation of calcium dependent PKC isoforms specifically PKCα. Addition of a PKCα inhibitor under these conditions most likely causes an increase in myofilament sensitivity enhancing contraction.

This was not seen with the lower concentration of APC (5nM) with the PKCαI. The possible explanation here is that in the absence of any significant calcium rise, PKCα activation is negligible and therefore the role of the inhibitor is minimal. The likely mechanistic framework describing the above is summarised in Figure 6.4.

In contrast to PKCα knockout mice, no effect on shortening was seen with the PKCαI alone. This may relate to differences between *in vitro* and *in vivo* models or levels of PKCα activation in the absence of a stimulus such as APC or thrombin.

Distinction in PAR-1-dependent responses between APC and thrombin make this cardiomyocyte model appropriate for studies aimed at distinguishing cytoprotective from cytodestructive signalling pathways. In particular, the data support previous suggestions that higher concentrations of APC exhibit characteristics similar to low

dose thrombin, where concentration determines which specific signalling pathway ensues [317].

Overall, the results from this chapter support our initial findings in Chapter 4 of the bell shaped concentration response curve for increasing [APC]. Up to the peak effect on shortening seen with 5nM APC, we had proposed an increase in myofilament sensitivity as no significant rise in $[Ca^{2+}]_i$ had been detected. From 5-50nM APC although $[Ca^{2+}]_i$ was rising, the shortening effect of APC was diminishing suggesting a fall in myofilament sensitivity. Figure 6.5 summarises the role of the PKCαI when used in either half of the bell shaped concentration response curve for increasing [APC] and offers further understanding of the mode of action for APC's positive inotropic properties. Further delineation of PAR-1 mediated signalling pathways also provides novel therapeutic foci of how to augment cardiac contractility both in sepsis and other conditions of cardiac compromise. Studies aimed at defining such pathways are an area of future work.

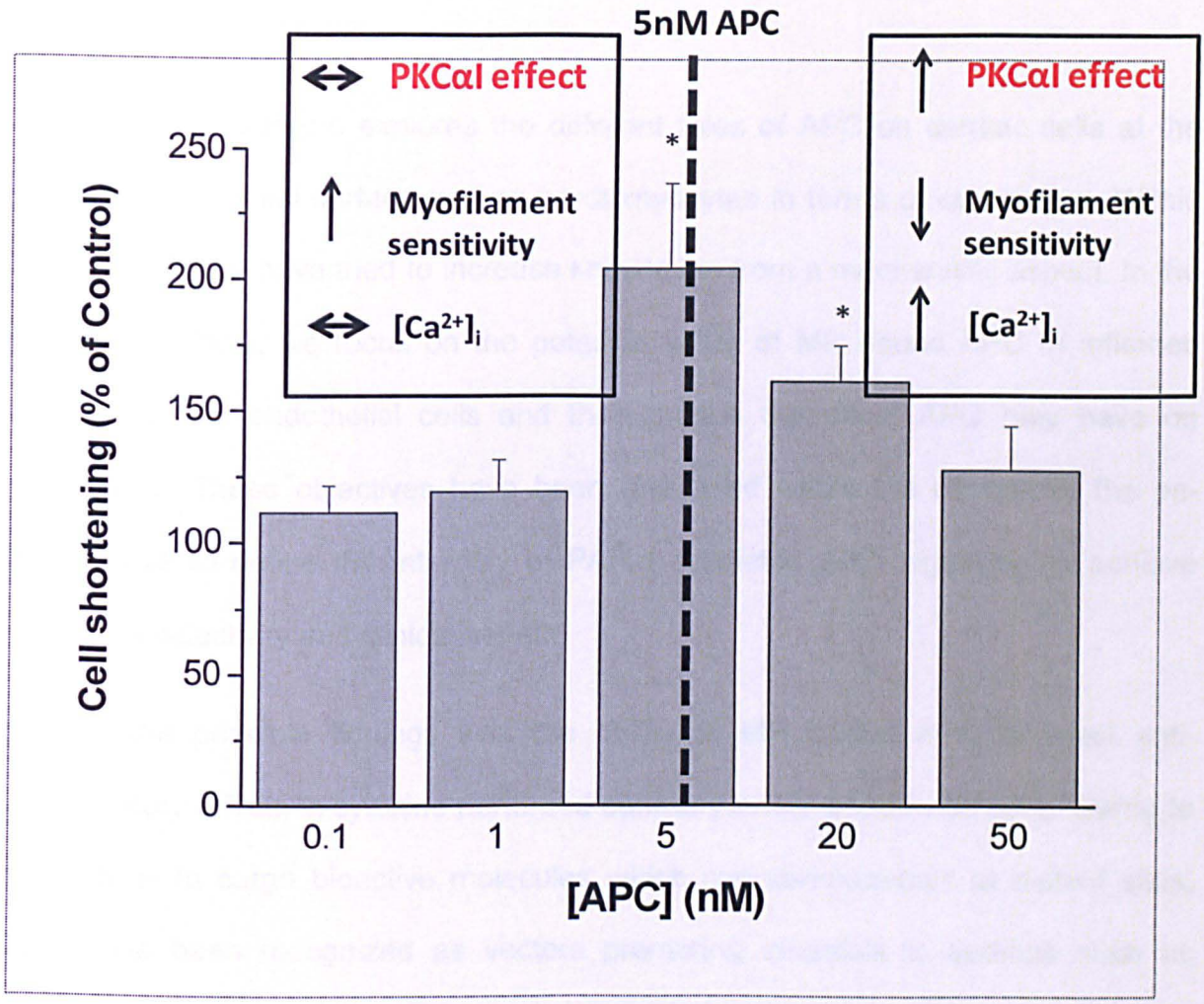

Figure 6.5 Schematic diagram proposing the role of the PKCαI to account for APC induced changes in cardiomyocyte shortening and $[Ca^{2+}]_i$ .An increase in PKCαI action is associated with an increased effect on shortening. This is observed for APC 50nM (right side of dotted line) but not APC < 5nM where no significant rise in $[Ca^{2+}]i$ is induced.

Chapter 7 General Discussion and Future Work

7.1 General Discussion

The work of this thesis explores the different roles of APC on cardiac cells at the coronary endothelial surface and on cardiomyocytes in terms of contraction. Within our approach we have tried to increase knowledge from a mechanistic aspect. In the context of sepsis, we focus on the potential value of MP bound APC in inflamed coronary artery endothelial cells and then pursue the effect APC may have on contractility. These objectives have been embraced within the context of the on-going strive to define the interplay of PAR-1 mediated APC signalling to achieve increased selectivity and clinical benefit.

One of the principle findings was the ability of MP bound APC to exert anti-inflammatory effects in cytokine perturbed coronary artery endothelial cells. Owing to their ability to cargo bioactive molecules which can communicate at distant sites, MPs have been recognized as vectors promoting crosstalk in settings such as inflammation. Typically they have been described as procoagulant in nature and a source of pro-inflammatory effectors [318]. In our work, we show that APC stimulates the release of EPCR-APC bound MPs from HCAECs and that application of these MPs to inflamed HCAECs results in an anti-inflammatory rescue effect. These results have important implications for the role of MPs in transcellular communication as it has been reported that EPCR is not widely distributed throughout tissues and vasculature. Now that we have shown that the APC bound to MPs exhibits anti-inflammatory properties comparable to free APC, circulation of these MPs to EPCR deficient site offers a potential delivery system that could be exploited pharmacologically.

The other principle finding from our work was the positive inotropic effect of APC demonstrated at the cellular level. This action of APC has not previously been described and of particular interest, the effects were observed at physiologically relevant APC concentrations. The dosing regime in the therapeutic application of APC in sepsis remains to be optimized and studies exploring differential dosing regimens are on-going [319, 320]. We report peak effects on cardiomyocyte contraction close to therapeutically relevant ranges at 5nM APC. Patients who reportedly benefit most from APC treatment are those with the most depressed PC levels and this can be related to our findings [229, 275]. Similarly the finding that low risk septic patients do not derive benefit from APC treatment might be because treatment leads to levels of APC that overshoot our observed window of positive cardiomyocyte-inotropic effect [321]. With a basis in sepsis, we have suggested that this could have relevance to the therapeutics of myocardial depression in general, in particular with the development of non-anticoagulant APC variants, which retain EPCR-PAR-1 signalling [274, 295]. This presents an area in which our work could be extended further exploring whether such variants demonstrate inotropic properties. The concentration response data we provide also presents a platform to investigate organ specific dosing and this is of relevance when considering causes of cardiac impairment other than sepsis, where there may be no underlying Protein C deficiency.

After demonstrating that APC has an effect on cardiomyocyte shortening, we show that this is dependent upon PAR-1 and further confirm the role of the APC-EPCR-PAR-1 axis by demonstrating the presence of both receptors. With the knowledge that calcium is required to elicit cardiac contraction we go on to show that APC also

induces a rise in cardiomyocyte $[Ca^{2+}]_i$. The rise in $[Ca^{2+}]_i$ is observed, however at relatively higher APC concentrations leading us to believe that the mechanism of action of APC's effects is based upon changes in myofilament sensitivity to calcium.

With reference to studies demonstrating that the activation of PAR-1 by thrombin and APC on cultured vascular endothelial cells elicits paradoxical proinflammatory and anti-inflammatory responses, respectively we studied the effect of APC on shortening in the presence of thrombin. In the approach to this part of our work, we characterized the effect of a range of thrombin concentrations on cardiomyocyte shortening, not previously defined in the literature. Thrombin was found to have PAR-1 dependent negative effects on cardiomyoyte function, however interestingly the effects of APC could still be detected using a lower thrombin concentration. This supports the work of Riewald *et al* reporting different cellular signalling for APC activation of PAR-1 versus thrombin despite cleaving the same site on the receptor, [248], and Scheupbach *et al* which showed that APC can induce powerful barrier protective responses in an endothelial cell monolayer in the presence of thrombin [76].

The final chapter looked at elucidating cellular signal pathways as a means of explaining the functional differences brought about through the activation of the same receptor. PAR-1 variants that are efficiently activated by APC but not by thrombin have been designed. Transgenic mice expressing these variants in endothelial cells have been generated and are currently analysed in sepsis models to define the *in vivo* roles of PAR-1 signalling. Availability of such models would allow us to pursue further support of the data and theories provided in this thesis.

Drawing our data together with insight in to possible signalling pathways from the literature we investigated a possible role for PKCα as a downstream mediator from PAR-1, reported to reduce contractility through reducing myofilament sensitivity. Using a PKCα inhibitor we were able to increase APC induced cardiomyocyte shortening, further supporting our belief that the APC effects were brought about by an increase in myofilament sensitivity. Based on these findings from the final chapters we can conclude that APC and thrombin can affect cardiomyocyte contractility and that the signalling pathways involved are PAR-1-PKC dependent with downstream effects on myofilament sensitivity. Whilst the caveat to the translational potential of our findings is that the observations are in healthy adult rat cardiomyocytes, our results suggest a mechanism at the single cell level to explain related findings in a modified Langendorff isolated heart preparation. Favory *et al* demonstrated that APC can reduce endotoxin-induced cardiac inflammation and improve left ventricular developed pressure [197]. To further these with our own findings, application of APC in a septic whole animal model with cardiovascular output measurement would provide valuable associated data.

APC has recently been withdrawn from commercial use for the treatment of patients with severe sepsis due to uncertainty regarding its risk/benefit profile. An increased bleeding risk had been noted in the ENHANCE (Extended Evaluation of Human Recombinant Activated Protein C) open-label study (6.5% serious bleeding and 1.5% intracranial haemorrhage) [177], and sub-analysis of the ADDRESS trial designed to examine the role of APC in those with severe sepsis and a low risk of death showed a higher 28 day mortality in the severe sepsis group [178], the cohort in which its initial benefits had been highlighted in the Prowess Trial. Re-examination of the risk/benefit profile of APC was consequently instructed by the Committee for

Medicinal Products for Human Use section of the European Medicines Agency (EMA) and this led to the placebo- controlled trial, named PROWESS–SHOCK commenced with a primary objective of assessing all-cause mortality at 28 days. Although the risk of severe bleeding events was 1.2% in the APC arm and 1.0% in the placebo arm, suggesting there was no increased harm, the trial failed to show any statistically significant difference in the mortality rate for placebo versus APC treated patients. The manufacturers of APC have since withdrawn their product from the market worldwide and both the EMA and the US Federal Drug Administration Agency have advised physicians not to initiate treatment with APC in new patients and to stop on-going treatment. Some have suggested, however, differences in mortality comparing the original Prowess Trial with the latest Prowess-Shock relate to improvements in the general care for patients with sepsis, and consequently the benefits of APC treatment have now been negated.

Nevertheless the therapeutic value of APC continues to be studied and recognized across multiple disease frontiers including pancreatitis [322], trauma [323], intestinal inflammation [324], acute respiratory distress [325], and pneumonia [326] in addition to further studies describing the beneficial effects of rhAPC on coronary injury [327-329]. To improve on the benefit-risk ratio of APC treatment in sepsis, knowledge of the mechanisms involved *in vivo* would be highly relevant and therefore work in this field remains valuable and a means of translating the diverse properties of APC in to safe patient care.

7.2 Future Work

The results presented in this thesis are novel and provide the foundation for significant further work in this area.

The first experimental chapter provides data to support the relevance of EPCR in inflamed coronary vasculature. MP bound APC demonstrated anti-inflammatory properties comparable to free APC however limitations were experienced with respect to the cytokine stimulated cellular model of sepsis. To overcome these we could test the effect of MP APC using an animal model of sepsis. With access to patient research samples from the ITU, we could do these using MPs from APC treated patients. With persistent concerns regarding the bleeding risk profile of APC this area of work remains of value.

The positive inotropic properties of APC have not been previously reported and are of translational value. The final experimental chapter begins to provide mechanistic reasoning in to these new findings in relation to literature already published in this area.

5nM APC was shown to significantly increase cardiomyocyte shortening without increasing $[Ca^{2+}]_i$. It was postulated that this effect was mediated by an increased sensitivity of cardiac myofilaments to calcium. This likely theory led to exploring a mechanism for the observed effects with the knowledge that activation/over-expression of PKCα, a downstream effector of PAR-1 signalling, adversely affects cardiomyocyte contractility [312] [315].

Cardiomyocyte contractility is regulated by $[Ca^{2+}]_i$ [330] and alterations in myofilaments properties [331] working together or independently. Phosphorylation of

the inhibitory unit of the cardiac troponin I (cTI) affects the myofilaments status and its responsiveness to intracellular calcium, which in turn affects the force of contraction (inotropy) and other contractile parameters of cardiomyocytes [330]. cTI has several phosphorylation sites controlled mainly by protein kinase C (PKC) and as previously described PKC is known to be situated downstream of G protein-coupled receptors such as PAR-1.

To explore the hypothesis that the inotropic effect of 5nM APC occurs through a reduction in myofilament sensitivity, one could investigate the effect of APC on PKCα activation in cardiomyocytes, and on cTI phosphorylation. Based on the data so far, we would hypothesize that APC and thrombin can alter cTI phosphorylation status via a PAR-1-PKC dependent pathway and therefore affect cardiomyocyte contractility.

The experimental procedure to study this would be based upon the knowledge that PKCs generally translocate from the cytosol to the membrane compartments of cells when activated [332].

PKC activation could be tested by performing sub-cellular fractionation (to separate the cytosolic from the membrane compartments of the cells) and then subjecting the different fractions to western blotting analysis. Cardiac troponin I phosphorylation could be assessed at the T143 phosphorylation site using western blotting. This site is PKC-dependent and its phosphorylation would lead to reduced sensitivity of the cardiac myofilments to calcium and reduced sliding velocity which ultimately leads to a negative inotropic effect [224].

To pursue the translational value of the work done here so far, the next step would be to study the effects of infused APC in an animal model of sepsis. Using a rodent

model of sepsis we would hypothesise that APC would have favourable effects on measured cardiovascular output parameters including mean arterial pressure (MAP), ventricular contraction rates, left ventricular ejection force and the need for inotropic support.

To confirm our findings relating to PKCα inhibition we could also use PKCα knockout mice. This would also overcome possible cross selectivity of the PKC isoforms seen at concentrations greater than what was used in our experiments.

As previously suggested the positive inotropic effects of APC can be more generally related to conditions in which failure of myocardial function is a feature. These could include cardiomyopathies, congestive cardiac failure, arrhythmias and therefore the potential scope of application ultimately is wide.

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