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Sec. Research Graduate School Committee

ACTIVATION OF HUMAN PROTEASE-ACTIVATED RECEPTORS BY PROTEASES FROM A PERIODONTAL PATHOGEN

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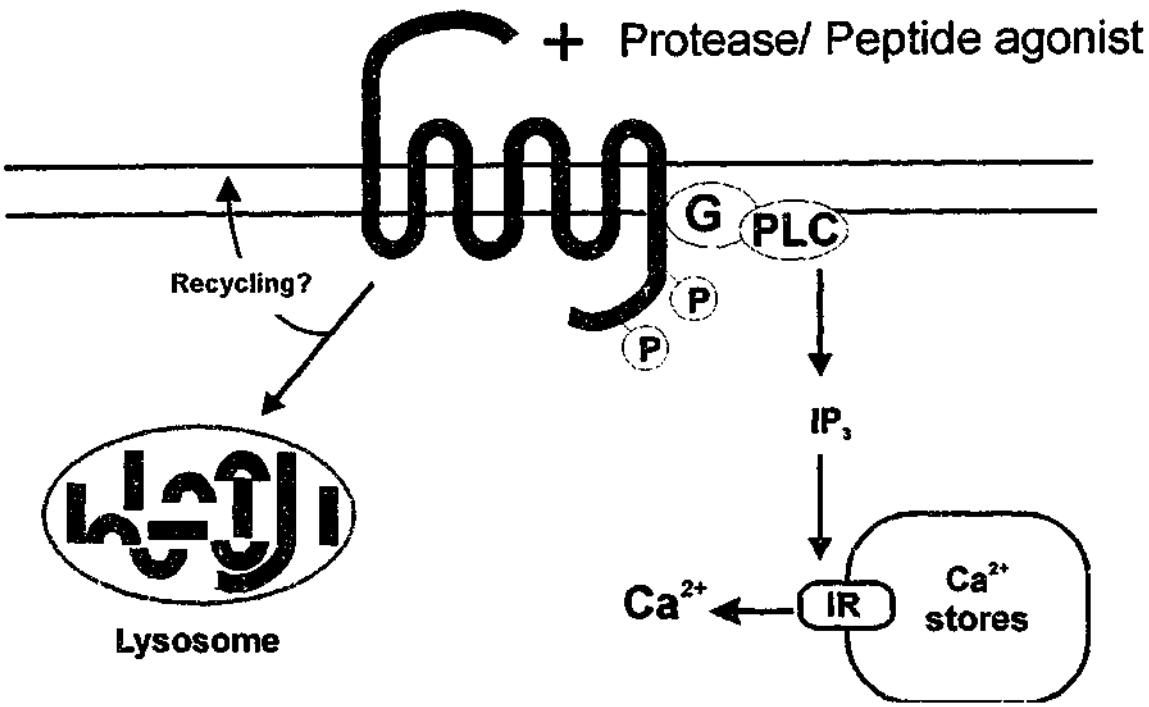
To Stavroula, George and Eleni Lourbakos

ERRATA

Chapter 1

Fig. 1.3 The text alludes to the possibility that signals from PARs might be shut off due to degradation of the receptors in lysosomes. It is also possible that a proportion of the receptors will be recycled, therefore the figure has been modified to incorporate this possibility.



Page 13, lines 1 and 3, Ga_0 should be $G\alpha_0$, referring to the g-protein "G alpha zero".

Figure 1.5 is from the web page of Dr. Walter Loesche (University of Michigan School of Dentistry (http://loeschelabs.dent.umich.edu).

Figure 1.6 is adapted from the web page of Dr. David W. Lucht (http://users.erols.com/dlucht/first.htm).

Figure 1.7 is from the *Porphyromonas gingivalis* Genome Project web page (http://www.pgingivalis.org/ATCC33277(2).htm).

Chapter 3

With regard to the activation of PAR-2 on neutrophils in the context of Periodontal disease, it should be noted that this would most likely occur on neutrophils which had already been primed and activated. It is therefore possible that the approach taken in the study, i.e. to use non-activated populations of neutrophils from donors, would not be perfectly in context in terms of the responses observed. Future studies might take this into account by artificially activating the neutrophils *in vitro* prior to experimentation.

With regard to the lack of response to trypsin or RgpB in some populations of neutrophils, it is unknown at present why this occurs. There is no evidence that it is due to PAR-2 binding proteins being present, which might therefore protect the receptor from cleavage in these populations. It is unlikely to be due to the presence of inhibitors to RgpB, since trypsin was also ineffective and also RgpB is reasonably resistant to inhibition by most host protease inhibitors.

With regard to the cleavage position of RgpB on PAR-2: activation of the PARs, as detected by subsequent intracellular calcium responses, only occurs when the receptor is activated at the specific position required to expose the new N-terminus, which then acts as the tethered ligand for the receptor. This is a highly specific process which is critically dependent on activation at the correct position. Trypsin appears to only cleave PAR-2 at the correct position (after Arg 36), as does RgpB, since there is no evidence for receptor inactivation by these enzymes, which might occur if they were cleaving at incorrect positions in the N-terminus of the receptor. RgpB is specific for cleavage after arginine residues and thus it wouldn't be expected to cleave at all the positions which trypsin does, since the latter enzyme cleaves after both arginine and lysine residues.

Page 79, line 12: Table 1 should be Table 3.1.

Tables 3.3 and 3.4 show duplicate values for the measurements instead of using mean ± standard error due to the fact that only duplicate readings could be taken in the context of the experiments. In this context it is scientifically more correct (in the opinion of student and supervisors) to show both readings.

Table 3.4. **CD11b increase in response to various agonists in neutrophils**

The median fluorescence intensity for the peak shifted by CD11b antibodies in each experiment (see Fig 3.10) is shown in relation to a treatment with PAR-2 agonist peptide (RAP), RgpB and fMLP. The cysteine in the buffer for RgpB was shown to induce effects alone, therefore a separate control (cysteine buffer) is shown for this enzyme, in relation to the peptide agonists (RAP and fMLP) which did not require cysteine in the buffer.

CD11b Median Fluorescence intensity

Donor	**Control** cysteine buffer	**RgpB** 50 nM	**Control**	**RAP** 300 μM	**fMLP** 100 nM
1	nd	nd	108, 117	289, 327	697, 673
2	107, 100	237, 200	171, 159	486, 414	1333, 1000
3	13,13	29, 21	nd	nd	nd
4	62, 67	264, 171	110	nd	352, 327
5	16, 13	35, 25	14, 15	nd	25

Chapter 4

Page 123, line 12: protease activated receptor should be abbreviated to PAR.

Increased concentration of RgpB or longer periods of treatment by the enzyme appeared to cause detachment of the epithelial cells. This would most be due to cleavage of adherence molecules, not apoptosis of the cells, but this could be a mechanism of interest *in vivo*, although it must be acknowledged that the concentrations of RgpB which might occur pathophysiologically and the length of time for which they would be active is unknown.

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DECLARATION

The work described in this thesis was carried out in the Department of Biochemistry and Molecular Biology at Monash University. This work is entirely my own unless otherwise acknowledged. No part of this dissertation has already been, or is currently being submitted for any degree, diploma or qualification at this, or any other, university.

Afrodite Lourbakos

February 2001

PUBLICATIONS ARISING FROM THIS WORK

Lourbakos, A., Chinni, C., Thompson, P., Potempa, J., Travis, J., Mackie, E. J., and Pike, R. N. (1998) Cleavage and activation of proteinase-activated receptor-2 on human neutrophils by gingipain-R from Porphyromonas gingivalis. *FEBS Lett.* 435: 45-8

Abraham, L. A., Chinni, C., Jenkins, A., Lourbakos, A., Ally, N., Pike, R. N., and Mackie, E. J., (2000) Expression of protease-activated receptor-2 by osteoblasts. *Bone* 26(1): 7-14.

Lourbakos, A., Yuan, Y., Jenkins, A., Travis, A., Andrade-Gordon, P., Santulli, R., Potempa, J., Pike, R. N. Activation of protease activated receptors by gingipains from *Porphyromonas gingivalis* leads to platelet aggregation: a new trait in microbial pathogenicity. *Blood* (under consideration).

Lourbakos, A., Potempa, J., Travis, J., Mackie, E. J, Pike, R. N. An arginine specific protease from *Porphyromonas gingivalis* activates protease-activated receptors on human oral epithelial cells and induces interleukin-6 secretion. *Infect. Immun.* (submitted)

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Thanks are also due to Mr Ray Spark and Dr Harshal Nandurkar and Dr Cindy O'Malley for drawing blood, everyone that kindly donated blood, Anna Romeo for her help with the FACS analysis, Nafisa Ally for helping with the purification of RgpB, Mike De Niese and Dr Absorn Sriratana for their invaluable advice in tissue culture. I thank Assoc. Prof. Rod Devenish, Dr Mark Prescott, Maria Lim, James Irving and Mark Baker for their interest in my work. I thank: Lakshmi Wijeyewickrema for all her help and mischief, and Helen Kasimiotis and Penny Moutsoulas for their support and interesting discussions.

SUMMARY

Protease-activated receptors (PARs) are a family of cell surface receptors that are activated by cleavage of their extracellular N-terminus at a specific scissile bond by a protease. A new N-terminus is formed and acts as a tethered ligand, causing receptor activation. Synthetic peptides (agonists) corresponding to the tethered ligand activate PARs independently of cleavage by a protease. PARs can be activated by any protease that can cleave the extracellular domain at the unique site required for exposure of the tethered ligand. In order to fully elucidate the role of PARs in physiology and disease, the entire spectrum of proteases that activate these receptors needs to be identified. PARs might confer responsiveness not only to endogenous but also exogenous proteases.

A disease state in which human cells are exposed to exogenous proteases is periodontal disease. The bacterium *Porphyromonas gingivalis*, a major etiological agent in the periodontal disease process, produces cysteine proteases termed gingipains that are considered to be important virulence factors. Gingipains which solely hydrolyze peptide bonds after arginine residues are known as gingipains-R, and two soluble secreted forms of these enzymes are HRgpA and RgpB. The ability of these bacterial proteases to cleave and activate PARs on the surface of human cells potentially involved in periodontal disease was examined.

The protease RgpB was purified from *P. gingivalis* strain H66. RgpB cleaved a synthetic fluorescent quenched corresponding to the cleavage site of PAR-2, which is the only known PAR expressed on the surface of neutrophils. Neutrophils are known to be present in very high numbers in sites of periodontal disease and are therefore likely to come

into contact with *P. gingivalis*. RgpB induced calcium mobilisation in human neutrophils from donors that also responded to trypsin, a known activator of PAR-2. Pre-treatment with a phorbol ester abrogated the calcium signalling induced by the PAR-2 agonist peptide, trypsin or RgpB. Based on calcium desensitisation studies with trypsin and the PAR-2 agonist peptide, it appears that RgpB induced a $[Ca^{2+}]_i$, increase by activating PAR-2 on human neutrophils. The ability of RgpB to activate PAR-2 was verified by the finding that the bacterial protease activated PAR-2 in transfected cells stably expressing the receptor. It was found that treatment of neutrophils with the PAR-2 agonist peptide, trypsin or RgpB did not cause myeloperoxidase release, indicating that PAR-2 activation did not induce neutrophil degranulation. Stimulation of neutrophils with the PAR-2 agonist peptide and to a lesser extent with RgpB caused an increase in the expression of CD11b, a marker of neutrophil activation.

The KB60 cell line was used as a model to investigate the effect of RgpB on gingival epithelial cells, which may in effect be the first cellular barrier to come into contact with periodontal bacteria, such as *P. gingivalis*. It was determined by reverse-transcription PCR that KB60 cells express PAR-1, -2 and –3, but not PAR-4. PAR-1 and PAR-2 agonist peptides caused an increase in $[Ca^{2+}]_i$, whereas PAR-4 did not induce calcium mobilisation, verifying the expression of functional PAR-1 and PAR-2 on KB60 cells. Thrombin, a known activator of PAR-1, -2 and –4, and trypsin, which activates PAR-1 and -2, also caused an increase in $[Ca^{2+}]_i$. RgpB induced calcium mobilisation in these cells which was dependent on its proteolytic activity, and calcium desensitisation studies with trypsin and thrombin indicated that RgpB activated PAR-1 and PAR-2 in KB60 cells. This was supported by the finding that RgpB activated both PAR-1 and PAR-2 in transfected cells stably expressing these receptors. Treatment of KB60 cells with

PAR-1 or PAR-2 agonist peptides resulted in increase in interleukin-6 (IL-6) secretion, as did treatment with thrombin or trypsin. RgpB was also found to induce IL-6 secretion and this effect was mediated by its proteolytic activity, indicating that the bacterial protease induced secretion of the cytokine by activating PAR-1 and PAR-2.

The arginine-specific gingipains have previously been found to activate soluble enzymes involved in the coagulation cascade. To expand our knowledge on the interaction between gingipains-R and cellular components of the coagulation system the effects of RgpB and HRgpA on human platelets were examined. The enzymes induced an increase in intracellular calcium concentration in platelets and caused platelet aggregation with efficiency comparable to thrombin in all donors tested. Both effects were dependent on the proteolytic activity of the enzymes. Based on desensitization studies carried out with thrombin and peptide receptor agonists, and immunoinhibition experiments using an antibody that recognised the cleavage site of PAR-1, gingipains-R appeared to be activating PAR-1 and -4, which are expressed on the surface of platelets. This was confirmed by the finding that HRgpA and RgpB potently activated PAR-1 and PAR-4 in transfected cells stably expressing these receptors. Cumulatively, the data in this study provides a mechanism by which bacterial proteases act through human receptors to induce cellular responses that may underlie reported associations between periodontal disease and cardiovascular disease.

ABBREVIATIONS

ATP	adenosine triphosphate
BSA	bovine serum albumin
$[Ca^{2+}]_i$	intracellular calcium concentration
DAPI	4',6'-diamidino-2-phenylindole
DEPC	diethyl pyrocarbonate
dH_20	distilled water
ddH_20	deionised distilled water
DMSO	dimethyl sulphoxide
ELISA	enzyme linked immunosorbent assay
EM	extracellular medium
EDTA	ethylelenediaminetetra-acetate
FACS	fluorescence activated cell sorter
FCS	foetal calf serum
fMLP	N-formylmethionine-leucyl-phenylalanine
HEPES	N-(2-hydroxyethyl)-1-piperizine-N'-2-ethanesulphonic acid
hr	hour
INF-γ	interferon-γ
IL-	interleukin-
min	minute
MOPS	3-[N-Morpholino]propanesulfonic acid
PAR-	protease-activated receptor-
PBS	phosphate buffered saline
PE	phycoerythrin
PCR	polymerase chain reaction
PGI_2	prostacyclin
RAP	PAR-2 activating peptide
RT-PCR	reverse transcription - polymerase chain reaction
SDS-PAGE	sodium dodecylsulfate polyacrylamide gel electrophoresis
TRAP	thrombin receptor activating peptide
TRAP-4	PAR-4 activating peptide
TNF-α	tumor necrosis factor-α

CHAPTER 1

Introduction

1.0 Overview

Proteolytic modification of intra- and extra-cellular proteins has recently been recognized as an important and common mechanism of regulation of cell function (Tansey, 1999; Peschon *et al.*, 1998). Extracellularly, transformation of the cell surface through proteolysis plays a role in cell migration, wound healing and tissue remodeling (Blasi, 1999; Murphy & Gavrilovic, 1999). In addition, a cohort of biologically active compounds, including cytokines and cytokine receptors, growth factors and growth factor receptors, cell-surface adhesion molecules, Fc receptors and G-protein-coupled receptors, are known to be released from cell surfaces through proteolytic cleavage (Kiessling and Gordon, 1998; Mullberg *et al.*, 2000). Under physiological conditions, it has been well established that enzymes belonging to a family of tightly regulated metalloproteases, which contain a disintegrin domain (ADAM family), mediate this ectodomain shedding (Schlondorff & Blobel, 1999). However, an alternative mechanism that activates numerous cells normally operates through highly specified proteolysis of a new family of cell surface receptors, referred to as protease-activated receptors (PARs) (Coughlin, 1999). While both mechanisms are tightly controlled, during bacterial infections the surface proteins and receptors may become a target for non-host proteases, which generally escape any control by host inhibitors. Indeed, there is growing evidence which indicates that bacterial proteases can modify the host cell surface and thus contribute significantly to microbial

pathogenicity (Ijiri *et al.*, 1994; Vollmer *et al.*, 1996; Wolf *et al.*, 1994; Lala *et al.*, 1994; Jagels *et al.*, 1996; Scragg *et al.*, 1999; Katz *et al.*, 2000; Sugawara *et al.*, 2000).

Periodontal disease, the chronic infectious disease in which uncontrolled proteolytic activity derived from both host and bacteria plays a significant role in the destruction of tooth-supporting tissues, including the alveolar bone, is the most prevalent inflammatory disease in the world. Approximately 15% of the population suffers from its most severe form and, if untreated, it may result not only in tooth loss, but also in systemic complications (Offenbacher *et al.*, 1999; Beck *et al.*, 1996; Page, 1998; Schenkein, 1999). The major pathogens associated with periodontal disease are *Porphyromonas gingivalis, Bacteroides forsythus* and *Actinobacillus actinomycetemcomitans* (Newman, 1996). Amongst these, *P. gingivalis* has been identified as a major etiological agent in the pathogenesis of adult periodontal disease in humans (Newman, 1996; Slots *et al.*, 1986). This bacterium produces a number of powerful proteases, termed gingipains, which act as important pathogenic agents (Travis *et al.*, 2000). Gingipains that cleave specifically after arginine residues are termed gingipains-R.

Members of the protease-activated receptor (PAR) family are activated following cleavage after an arginine residue. The entire spectrum of proteases that activate PARs and induce intracellular signals in physiology and disease has not yet been elucidated (Coughlin, 2000). The hypothesis postulated is that the gingipains-R bacterial proteases are capable of activating protease-activated receptors expressed on the surface of human cells, with consequent induction of a number of cellular effects relevant to the pathogenesis of periodontal disease.

1.1 Protease-Activated Receptors (PARs)

Protease-activated receptors (PARs) are cell surface receptors that belong to a family of seven transmembrane domain G-protein-coupled receptors. PARs have a novel mechanism of activation as they carry their own ligands, which are revealed only following cleavage of their extracellular domain. The receptors are activated by cleavage of their extracellular N-terminus at a specific scissile bond by a protease (Vu *et al.*, 1991a; Nystedt *et al.*, 1994). The new N-terminus that is formed after receptor cleavage, acts as a tethered ligand that binds intramolecularly to the body of the receptor and leads to its activation (Vu *et al.*, 1991a; Vu *et al.*, 1991b; Chen *et al.*, 1994a). Specific residues within the tethered ligand, which consists of six or more amino acids, interact with extracellular domains of the receptor (Fig. 1.1).

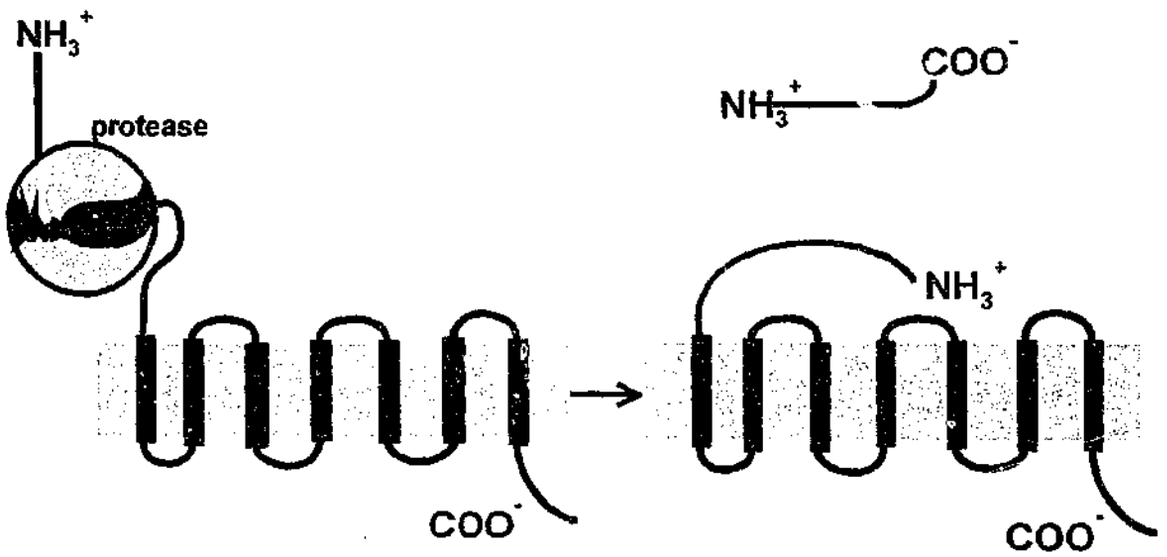


Figure 1.1. **A schematic representation of the mechanism of PAR activation.**
A protease (sphere) interacts with the N-terminal extracellular domain of a PAR and cleaves at a specific peptide bond to reveal a new N-terminus (red and green). The new N- terminus that is formed after receptor cleavage acts as a tethered ligand that binds intramolecularly to the body of the receptor. Specific residues within the tethered ligand (green), interact with extracellular domains of the receptor to effect transmembrane signalling.

To date, four protease-activated receptors have been identified: PARs-1, -2, -3 and -4. The extracellular domain of PAR-1 contains a cleavage site for thrombin (LDPR^{41}↓S^{42}FLLRN) in its extracellular domain and the new N-terminus, SFLLRN, activates the receptor. PAR-2 contains a putative cleavage site for trypsin (SKGR^{36}↓S^{37}LIGKV) and the tethered ligand domain, SLIGKV, activates the receptor. PAR-3 is cleaved at position, LPIK^{38}↓T^{39}FRG, and the new N-terminus, TFRGAP, was shown to be necessary for receptor activation. PAR-4 is cleaved at LPAPR^{47}↓G^{48}YPGQV to unmask the tethered ligand, GYPGQV (Fig. 1.2).

```
PAR-1   .T L D P R / S F L L R N P N D K Y E.  (TRAP)
PAR-2   .S S K G R / S L I G K V D G T S H V.  (RAP)
PAR-3   .T L I P K / T F R G A P P N S F E E.
PAR-4   .L P A P R / G Y P G Q V C A N D S D.  (TRAP-4)
                   ↑
             cleavage site
```

***Figure 1.2.* Sequence alignment of amino acids around the cleavage site of PARs.**
Synthetic peptides corresponding to the tethered ligand sequence (green) of PAR-1, -2 and -4, termed TRAP, RAP and TRAP-4 respectively, activate the receptors independent of receptor cleavage. Three of the known PARs are cleaved after an arginine residue.

Synthetic peptides (agonists) corresponding to the tethered ligand sequence of each receptor, with the exception of PAR-3, are able to activate the receptor, independent of receptor cleavage by a protease (Vu *et al.*, 1991a; Scarborough *et al.*, 1992; Nystedt *et al.*, 1994; Ishihara *et al.*, 1997; Kahn *et al.*, 1998a; Xu *et al.*, 1998) [Fig. 1.2]. A PAR-1 cleavage site mutant (R41A) receptor was not activated by thrombin, but responded to the PAR-1 agonist peptide, TRAP [SFLLRN] (Vu *et al.*, 1991a). Similarly, a murine PAR-2 cleavage site mutant (R34P) receptor no longer responded to trypsin but was responsive to

the murine PAR-2 agonist peptide [SLIGRL] (Nystedt *et al.*, 1994). A mutation of the cleavage site of PAR-4 (R47A) rendered it resistant to activation by thrombin, but still responsive to its agonist peptide, TRAP-4 [GYPGQV] (Xu *et al.*, 1998). The peptide agonists are weaker than the cognate protease activators. This difference in activating ability may be due to the inefficient presentation of these soluble peptides to the body of the receptor, compared with the tethered peptide. The cellular responses mediated by the different receptors can, however, be distinguished and studied by treatment of cells with their respective agonist peptides.

Studies of the structural basis of PAR activation have shown that it is determined by the extracellular surface of the receptors. The construction of chimeric receptors for PAR-1 and PAR-2 revealed that both the tethered ligand sequence within the new N-terminus and peptide agonists interact with the second extracellular loop, and that this loop is the primary determinant of agonist specificity (Lerner *et al.*, 1996; Al-Ani *et al.*, 1999; Gerszten *et al.*, 1994; Nanevicz *et al.*, 1995; Nanevicz *et al.*, 1996; Compton *et al.*, 2000). Mutagenesis studies have indicated that Arg^{5} residue in both the tethered ligand and the agonist peptide for human PAR-1 (SFLLRN) is recognized by the Glu^{260} residue in the second extracellular loop of the receptor (Nanevicz *et al.*, 1995). These residues are also conserved in the tethered ligand and agonist peptide (SLIGRL) and the second extracellular loop of murine PAR-2. The agonist peptide for human PAR-1 (SFLLRN) has also been found to activate murine PAR-2 (Nystedt *et al.*, 1994; Blackhart *et al.*, 1996), thus an Arg^{5}-Glu^{260} interaction might operate in recognition of SLIGRL by PAR-2 (Lerner *et al.*, 1996). The peptide agonists SFLLRN and SLIGRL exhibited equally decreased potency in activating a mutated murine PAR-2 in which the second extracellular loop sequence Pro^{233}-Glu^{234}-Glu^{235} was mutated to Pro-Arg-Arg, revealing that the Pro-Glu-Glu sequence

plays an important role in determining agonist specificity in murine PAR-2 (Al-Ani *et al.*, 1999). Mutation of the Pro-Glu-Glu sequence to Asn-Glu-Thr-Leu, which is homologous to the second extracellular loop sequence Asn^{259}-Glu^{260}-Thr^{261}-Leu^{262} in PAR-1, markedly reduced the activating ability of SFFLRN compared to SLIGRL. This suggests that SFFLRN and SLIGRL may interact in a distinct manner with PAR-2 and that SFLLRN may interact differently with PAR-2 than it does with PAR-1 (Al-Ani *et al.*, 1999).

In addition to the human PAR-2 agonist peptide (SLIGKV), the agonist peptides for human PAR-1 (SFLLRN) and murine PAR-2 (SLIGRL) have also been found to activate human PAR-2 and amino acid substitutions in the agonist peptides suggested that the amino-terminal serine residue is critical for PAR-2 agonist specificity (Blackhart *et al.*, 1996). A polymorphic form of human PAR-2, with a phenylalanine to serine mutation at residue 240 (F240S) within the second extracellular loop, displayed a significant reduction in sensitivity towards trypsin and the PAR-2-activating peptides, SLIGKV and SLIGRL, but an increased sensitivity towards the selective PAR-2 agonist, trans-cinnamoyl-LIGRLO and the selective PAR-1 agonist, TFLLR (Compton *et al.*, 2000). Furthermore, TLIGRL and a PAR-4 derived peptide, trans-cinnamoyl-YPGKF were found to be selective PAR-2 (F240S) agonists. Introduction of the F240S mutation into rat PAR-2 resulted in changes in agonist potencies that corresponded to the human PAR-2 (F240S), suggesting that F240 in the second extracellular loop is involved in determining agonist specificity of PAR-2 (Compton *et al.*, 2000).

Interactions between other extracellular domains of PARs probably also play a critical role in agonist recognition and receptor function. A chimeric PAR-1, in which all the cognate PAR-1 extracellular domains were substituted with PAR-2 extracellular domains, yielded a functional receptor with PAR-2-like specificity. However, substitution

of either the amino-terminal extracellular domain, or the third extracellular loop alone, with the cognate PAR-2 domains resulted in significant loss of both PAR-1 and PAR-2 function, yet the double substitution resulted in the recovery of PAR-2 specificity and function (Lerner *et al.*, 1996). This functional complementation produced by the double substitution was more significant if the second extracellular loop of PAR-2 was also included (Lerner *et al.*, 1996), indicating that a direct or indirect interaction of the amino-terminal extracellular domain and the third extracellular loop is required for PAR-1 and PAR-2 structure or function. Although the three-dimensional structure of G-protein-coupled receptors has not been solved at high resolution, Lerner *et al.* (1996) proposed an arrangement based on other models of G-protein-coupled receptor structure (Baldwin, 1993; Schertler *et al.*, 1993) in which the third extracellular loop is in close proximity to the amino terminal domain.

All PAR genes consist of two exons separated by a single intron with the cleavage site located within the second exon (Kahn *et al.*, 1998b). The PAR-1, -2 and -3 genes localise in the same region of the human genome at chromosome 5q13 (Bahou *et al.*, 1993; Schmidt *et al.*, 1997; Schmidt *et al.*, 1998), whereas the PAR-4 gene is present on chromosome 19p12 (Xu *et al.*, 1998) where a second cluster of PAR genes may be present (Kahn *et al.*, 1998b).

1.1.1 Protease agonists of the PARs

PAR-1 was the first receptor discovered and is cleaved and activated by thrombin (Vu *et al.*, 1991a; Rasmuussen *et al.*, 1991), as are PAR-3 and PAR-4, the two other thrombin receptors discovered more recently (Ishihara *et al.*, 1997; Schmidt *et al.*, 1998; Kahn *et al.*, 1998a; Xu *et al.*, 1998). PAR-1 and PAR-3 have sequences both N- and C-terminal to the cleavage site that resemble sequences with which thrombin interacts (Vu *et al.* 1991b; Liu *et al.*, 1991). An acidic sequence C-terminal to the cleavage site corresponds to a region of the leech anticoagulant, hirudin, which binds to thrombin's fibrinogen binding exosite. Interestingly, PAR-4 does not have this hirudin-like sequence and higher thrombin concentrations are necessary for its activation compared to the other thrombin receptors (Xu *et al.*, 1998; Kahn *et al.*, 1998a). However, mutagenesis studies in which thrombin's cleavage site in PAR-1 was replaced with the recognition site for the protease, enterokinase, resulted in complete change of the specificity of the receptor from a thrombin to an enterokinase receptor (Vu *et al.*, 1991b). This proved that proteolysis at the cleavage site of PAR-1 is sufficient for receptor activation (Coughlin, 2000). In addition, other proteases have been found to activate the "thrombin" receptors, such as trypsin which activated PAR-1 and PAR-4 [albeit with lower efficiency than thrombin] (Vu *et al.*, 1991a; Vouret-Craviari *et al.*, 1995; Parry *et al.*, 1996; Molino *et al.*, 1997a; Nystedt *et al.*, 1994; Xu *et al.*, 1998), granzyme A which was found to activate PAR-1 (Suidan *et al.*, 1994; Suidan *et al.*, 1996), and more recently cathepsin G, which was reported to activate PAR-4 (Sambrano *et al.*, 2000). All of these proteases activate PARs because they are capable of cleaving at the unique cleavage site required for exposure of the tethered ligand, which is sufficient for receptor activation.

Physiological activators of PAR-2 in many of the tissues where the receptor is highly expressed have not yet been defined. Initially, PAR-2 was identified as a trypsin receptor as it was activated by trypsin but not by thrombin (Nystedt *et al.*, 1994). Latterly, mast cell tryptase was also found to activate PAR-2 (Molino *et al*, 1997b) and may be important in inflammatory conditions. More recently, coagulation factors VIIa and Xa (Camerer *et al.*, 2000) and a novel protease termed membrane-type serine protease 1 (Takeuchi *et al.*, 2000) have also been found to activate PAR-2.

1.1.2 PAR Desensitization

PAR desensitization involves activation of the receptor by cleavage, termination of signalling and internalization of the receptor. PAR activation involves cleavage and is therefore an irreversible event, with the tethered ligand that is formed remaining attached to the body of the receptor. An intact N-terminus is necessary for activation by proteases, thus once a PAR is cleaved by a protease it can no longer be cleaved by the same or another protease, which makes PARs single use receptors.

Once PARs are activated they are then rapidly uncoupled from G-protein signalling. PAR-1 is uncoupled from signalling by phosphorylation-dependent mechanisms, as shown in transfected rat fibroblasts and HEK 293 cells (Vouret-Caviari *et al.*, 1995a; Hammes *et al.*, 1999). Termination of PAR-1 signalling is mediated by rapid phosphorylation of serine and threonine residues between 391 and 406 in the receptor's cytoplasmic carboxyl tail by G-protein-coupled receptor kinases (Ishii *et al.*, 1994; Vouret-Caviari *et al.*, 1995b; Shapiro *et al.*, 1996; Hammes *et al.*, 1999).

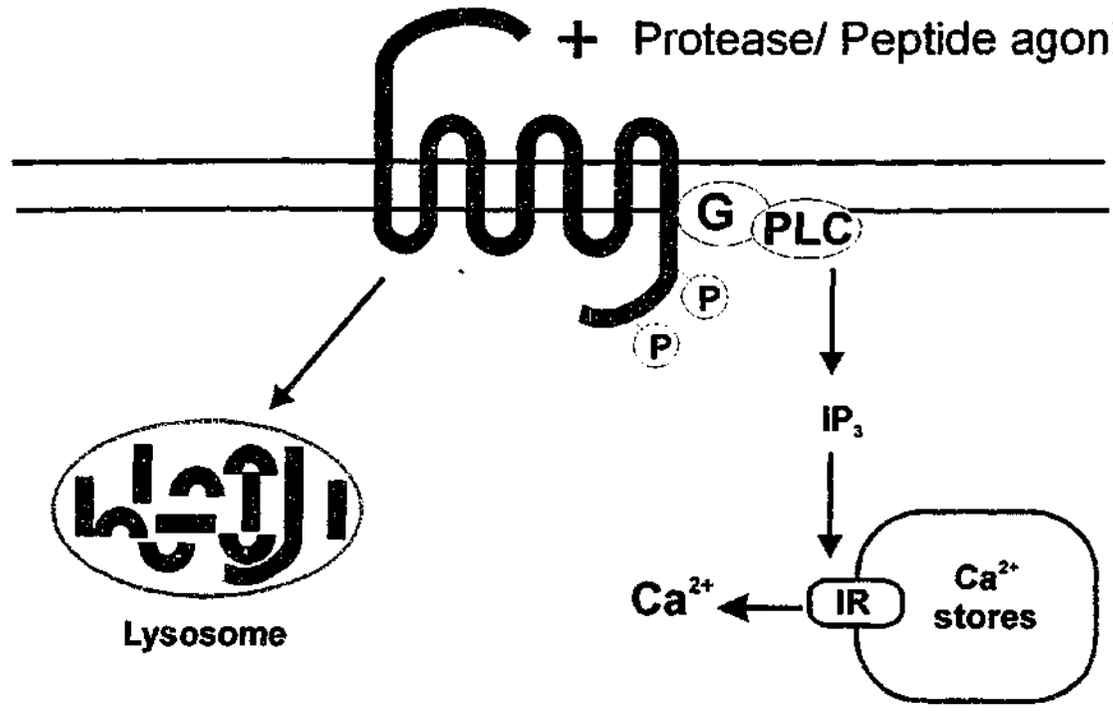


Figure 1.3. **Schematic representation of intracellular signalling and trafficking of PAR-1 following activation.**

Upon activation , PAR-1 couples to G proteins (Gq) to activate phospholipase C (PLC) inducing inositol 1,4,5-triphosphate (IP_3) production. IP_3 activates its receptor releasing calcium from stores to the cytoplasm. Following activation PAR-1 is phosphorylated, uncoupled from signalling, rapidly internalised and sorted into lysosomes where it is degraded.

Following activation by thrombin or the agonist peptide, the majority of PAR-1 molecules are removed from the surface of the cell (Brass *et al.*, 1993; Brass *et al.*, 1994; Hoxie *et al.*, 1993). Similarly, PAR-2 was cleared from the surface of endothelial cells, intestinal epithelial cells and kidney epithelial cells following proteolytic or non-proteolytic activation (Molino *et al.*, 1997a; Bohm *et al.*, 1996a). The majority of the activated PAR-1 molecules are rapidly internalised (enter endosomal compartments) and then sorted into lysosomes for degradation (Fig. 1.3), again through phosphorylation of its cytoplasmic tail at sites different from those required for receptor uncoupling from signaling (Hein *et al.*, 1994; Shapiro *et al.*, 1996; Hoxie *et al.*, 1993; Hammes *et al.*, 1999; Trejo *et al.*, 1998).

Similarly, following proteolytic or non-proteolytic activation PAR-2 is internalised and then targeted to lysosomes (Bohm *et al.*, 1996a; Dery *et al.*, 1999). Unlike other G-protein-coupled receptors that are recycled to the cell surface, it has been found that only a small portion of the internalised PAR-1 returns to the cell surface (Hoxie *et al.*, 1993; Brass et al., 1994). These receptors can be re-activated by the PAR-1 activating peptide, but not by protease agonists (Hoxie *et al.*, 1993; Brass, 1992). Thus PARs are used only once and then discarded. The internalization and degradation of PARs ensures that they are removed from the cell surface and prevents recycling to the cell surface, thus inhibiting continual signalling by an activated receptor (Trejo *et al.*, 1998). It has been found that phosphorylation of G-protein-coupled receptors promotes binding of the receptor to β-arrestins. Binding of G-protein-coupled receptors to β-arrestin prevents further interactions with G-proteins and also mediates receptor internalisation (Ferguson *et al.*, 1996). Recently, the trafficking of PAR-2 and β-arrestin 1 tagged with green fluorescent protein was studied, and revealed that arrestins mediate endocytosis of PAR-2 (Dery *et al.*, 1999; DeFea *et al.*, 2000).

Subsequent to internalization of used receptors, new receptors are delivered to the cell surface by mobilisation of intracellular pools of receptors or by protein synthesis. New PAR-1 molecules in endothelial and fibroblast cells (Hein *et al.* 1994; Horvat *et al.*, 1995) and new PAR-2 receptors in endothelial cells, intestinal epithelial cells and kidney epithelial cells stably expressing PAR-2 (Molino *et al.*, 1997a; Bohm *et al.*, 1996a), repopulate the cell surface over a period of 30 min to 2 hrs, from a preformed intracellular pool by a process that does not require protein synthesis. Yet in human megakaryocyte-like cell lines, recovery of PAR-1 to the cell surface requires protein synthesis (Brass, 1992; Hoxie *et al.*, 1993). The mode of exposure of new receptors to the cell surface may be

dependent on the cell type and the function of the receptor. For example, in platelets the majority of PAR-1 is on the plasma membrane and, following treatment with thrombin, the cells no longer respond to thrombin as they lack an intracellular pool of receptors (Molino *et al.*, 1997c). PAR-1 activation induces platelet aggregation and thus subsequent incorporation into thrombi, where PAR-1 stimulation may serve no further purpose (Coughlin, 2000).

1.1.3 PAR signalling

PAR-1, -2, -3, -4 couple to G-proteins to induce intracellular calcium mobilization and phosphoinisitol hydrolysis (Vu *et al.*, 1991a; Nystedt *et al.*, 1994; Bohm *et al.*, 1996b; Ishihara *et al.*, 1997; Faruqi *et al.*, 2000; Xu *et al.*, 1998; Kahn *et al.*, 1998a). To date, most of the research into the signalling pathways of PARs has focused on elucidating the signalling mechanisms of PAR-1.

PAR-1 couples to $G\alpha_q$ to activate phospholipase C inducing phosphoinositide hydrolysis, which causes an increase in intracellular calcium levels (Fig. 1.3), and diglyceride generation and subsequent activation of protein kinase C (Hung *et al.*, 1992; Benka *et al.*, 1995; Offermans *et al.*, 1994; Baffy *et al.*, 1994; Post *et al.*, 1996; Vouret-Craviari *et al.*, 1995) [Fig. 1.3]. Generally $G\alpha_q$ subunits activate phospholipase Cβ [PLCβ] (Taylor *et al.*, 1991) and PAR-1, through $G\alpha_q$, is also thought to activate PLCβ. Thrombin-induced inositol phosphate production was inhibited in a cell line expressing low levels of PLCβ but normal levels of other phospholipases (Fee *et al.*, 1994). PAR-1 activation also stimulates phospholipase A_2 in platelets (Seiler *et al.*, 1995) and endothelial cells (Molino *et al.*, 1997a).

Antibodies to Ga_o were found to inhibit PAR-1-induced calcium mobilisation and mitogenesis in chinese hamster lung fibroblasts (Baffy *et al.*, 1994), indicating that PAR-1 can also couple to Ga_o. PAR-1, via coupling to G_i, inhibits adenyl cyclase which supresses formation of cyclic AMP (Hung *et al.*, 1992), an event that promotes responses in platelets. Expression of a mutant $G\alpha_{12}$ inhibits thrombin stimulation of cytoplasmic phospholipase A_2-mediated arachidonic acid release (Winitz *et al.*, 1994). In astrocytoma cells and in cells co-expressing PAR-1 and $G\alpha_{12}$, PAR-1 couples to $G\alpha_{12}$ to induce Ras-dependent activating protein 1-mediated transcription and DNA replication (Aragay *et al.*, 1995; Post *et al.*, 1996). In platelets PAR-1 also couples to G_{12} and G_{13} (Offermans *et al.*, 1994), which can bind guanine-nucleotide exchange factors (RhoGEFs) that activate G-proteins such as Rho (Kozasa *et al.*, 1998; Hart *et al.*, 1998; Fukuhara *et al.*, 1999). Activation of G_{12} and G_{13} in platelets results in shape change and rho/rho-mediated myosin phosphorylation (Klages *et al.*, 1999) which may mediate PAR-1 induced platelet shape changes. In addition, Rho and Rho kinase mediate PAR-1 induced proliferation and migration of vascular smooth muscle cells (Seasholtz et al., 1999). PAR-4 couples to G_q, but not G_i (Faruqi *et al.*, 2000). PAR-2 stimulation caused activation of PLCβ to induce phosphoinisitol hydrolysis, increase in intracellular calcium levels as well as phospholipase A_2 activation, leading to PGI_2 release in endothelial cells (Molino *et al.*, 1997a) and prostaglandin E_2 and $F_1\alpha$ in epithelial cells (Kong *et al.*, 1997).

Phorbol myristate acetate, an activator of protein kinase C (PKC), inhibited thrombin-induced calcium mobilization and phosphoinisitol hydrolysis in human platelets (Zavoico *et al.*, 1985). Phorbol myristate acetate also inhibited PAR-1 calcium signalling in PAR-1 transfected oocytes and in HEL cells (Brass, 1992; Ishii *et al.*, 1994), suggesting that desensitisation of PAR-1 was the result of PKC phosphorylation of the receptor.

However, phorbol myristate acetate can also block the release of calcium from intracellular stores by inhibiting the inositol triphosphate (IP_3) production pathway through its effect on phospholipase C (PLC) (Smith *et al.*, 1987; Watson and Lapetina, 1985). It has been shown that PKC can phosphorylate PLCβ (Ryu *et al.*, 1990) and the α subunits of certain G- proteins (Bushfield *et al.*, 1990; Carlson *et al.*, 1989). In endothelial cells protein kinase Cβ (PKCβ) controlled the loss of PAR-1 cell surface expression following treatment with a PKC activator, but not by thrombin (Yan *et al.*, 1998), showing that PKCβ activated pathway inhibits PAR-1 mediated calcium signaling and regulates heterologous desensitisation of PAR-1. Yet co-expression of PAR-1 with G-protein-coupled receptor kinase 3 prevented thrombin-mediated signalling, suggesting that G-protein-coupled receptor kinase 3 plays a dominant role in homologous desensitisation of PAR-1 (Ishi *et al.*, 1994). It is possible that PKC terminates PAR-1 signalling by phosphorylating the receptor along with other proteins in the signalling pathway.

Termination of PAR-2 signalling was also found to be mediated by PKC. Acute activation of PKC decreases the calcium mobilisation in response to trypsin and RAP activation of PAR-2 in intestinal epithelial cells and kidney epithelial cells expressing PAR-2 (Bohm *et al.*, 1996a), whereas inhibition of PKC increases the calcium responses to trypsin and RAP. PAR-1 has several serine and threonine residues in its carboxyl terminus that are G-protein-coupled receptor kinase consensus sites, whereas PAR-2 has only one serine residue in the third intracellular loop that is a potential target for a G-protein-coupled receptor kinase. Yet there are a number of PKC sites in the carboxyl terminus of PAR-2, suggesting that PKC may play an important role in the homologous desensitisation of PAR-2 (Bohm *et al.*, 1996a).

Phosphorylation of mitogen activated protein (MAP) kinases is involved in activation of transcription factors and expression of genes that promote cell proliferation (Gille, 1991). In the case of the epidermal growth factor receptor, a phosphotyrosine in the intracellular domain is responsible for recruiting the Grb complex to exchange GDP for GTP on the small G-protein $p21^{ras}$. $p21^{ras}$ phosphorylates Raf-1 kinase, which then phosphorylates MAP kinase kinase, which finally phosphorylates MAP kinase. PAR-1 has been found to activate Raf kinase and MAP kinases in various cell types (Molloy *et al.*, 1996; Malarkey *et al.*, 1996; Seasholtz *et al.*, 1999; Vouret-Craviari *et al.*, 1993; Maruyama *et al.*, 1997). The mitogenic effect of PAR-1 is thought to be mediated by activation of MAP kinases. PAR-1 activation caused phosphorylation of Shc, which is recruited to Grb2 complexes (Chen *et al.*, 1996). Expression of mutant Shc proteins defective in Grb2 binding prevented PAR-1-induced activation of MAP kinase, gene induction and cell growth (Chen *et al.*, 1996). In mouse lung fibroblasts from a PAR-1 knockout mouse it was found that PAR-1 was necessary and sufficient for thrombin-induced MAP kinase activation and mitogenesis (Trejo *et al.*, 1996). Similarly, PAR-2 activation stimulated the activation of MAP kinase isoform p38 in vascular smooth muscle cells (Belham *et al.*, 1996), induced activation of MAP kinase isoforms ERK1/2 and regulated intracellular targeting of activated ERK1/2 (DeFea *et al.*, 2000).

PAR-1 was also found to activate tyrosine kinases Src and Fyn (Chen *et al.*, 1994b). Activation of PAR-1 and PAR-2 induced c-fos gene expression and phosphorylation of the tyrosine phosphatase SHP2 (Src homology 2 domain protein tyrosine phosphatase) which was shown to mediate the mitogenic effect of the receptors (Yu *et al.*, 1997; Rivard *et al.*, 1995; Yalkinoglu *et al.*, 1995).

1.1.4 Functions of PARs in physiological and pathophysiological conditions

The function of PARs appears to be dependent upon the cell type in which they are expressed.

1.1.4.a PAR-1

Haemostasis

PAR-1 has a wide tissue distribution and is expressed at high levels on cells essential to haemostasis such as platelets, endothelial cells and vascular smooth muscle cells. PAR-1 activation in human platelets induces platelet secretion and aggregation (Vu *et al.*, 1991a; Kahn *et al.*, 1998a) and this receptor is thought to mediate activation of human platelets to very low concentrations of thrombin (Kahn *et al.*, 1999). An antibody to the thrombin-binding exosite of PAR-1 inhibited experimental arterial thrombosis in the African green monkey (Cook *et al.*, 1995). Platelets play a central role in arterial thrombosis, thus PAR-1 activation has attracted immense interest as a target molecule for the design of antithrombotic drugs. PAR-1 activation in vascular endothelial cells resulted in release of von Willebrand factor (Storck *et al.*, 1996) and P-selectin (Sugama amd Malik, 1992), which may promote binding of platelets to the endothelial cell surface and affect haemostasis.

Inflammation

It has been suggested that PAR-1 may be involved in inflammation as it has been shown to affect vascular tone and permeability and facilitate the influx of leukocytes. PAR-1 may affect blood vessel tone and blood flow as both thrombin and the PAR-1 agonist peptide induced both vasodilation and vasoconstriction through endothelium-dependent relaxation of blood vessels and contraction of vascular smooth muscle cells, respectively (Muramatsu *et al.*, 1992; Antonaccio *et al.*, 1993; Ku and Zaleski, 1993; Hwa *et al.*, 1996). In PAR-2-deficient mice, a selective PAR-1 agonist peptide caused an initial decrease in blood pressure and heart rate followed by an increase in blood pressure (Damiano *et al.*, 1999), which may be the result of vasodilation followed by vasoconstriction.

PAR-1 activation in vascular endothelial cells induced release of von Willebrand factor (Storck *et al.*, 1996), monocyte chemotactic protein-1, an important mediator of monocyte recruitment, (Colotta *et al.*, 1994; Grandaliano *et al.*, 1994) and P-selectin, which mediated endothelial hyperadhesivity and neutrophil adhesion (Sugama and Malik, 1992). Thus PAR-1 activation on endothelial cells may promote binding of platelets and leukocytes to the endothelial cell surface during vascular injury, through up-regulation of adhesion molecules. In addition, it was shown in an animal model that injection of PAR-1 agonist peptide or thrombin caused oedoma formation and extravasation of plasma proteins, which was partly mediated by biogenic amines released from mast cells (Cirino *et al.*, 1996). Exposure of the subendothelium may promote the formation of additional thrombin "as plasma coagulation factors (factor VIIa) contact extravascular tissue factors" (Coughlin, 2000), which may cause aggregation of platelets and subsequent thrombosis. Accumulated leukocytes, through their products, may directly injure the vascular tissue and further promote the inflammatory process. The role of PAR-1 in inflammation is supported

by the finding that PAR-1 deficency appears to be protective in a mouse model of glomerulonephritis (Cunningham *et al.*, 2000).

Mitogenesis

PAR-1 activation mediates mitogenic effects in endothelial cells (Mirza *et al.*, 1996; Schaeffer *et al.*, 1997), vascular smooth muscle cells (Kanthou *et al.*, 1995; McNamara *et al.*, 1993; Molloy *et al.*, 1996), fibroblasts (Van Corven *et al.*, 1993; Chen *et al.*, 1994b; Hollenberg *et al.*, 1996a), mesangial cells (Albrightson, 1994), uterine stromal cells (Arena *et al.*, 1996), osteoblasts (Abraham and Mackie, 1999) and keratinocytes (Derian *et al.*, 1997). Thus PAR-1 may contribute to the replacement of cells following inflammation or injury. Following vascular injury, PAR-1 may promote the accumulation of smooth muscle cells in vessels, which are one of the components of fibrous lessions associated with atherosclerosis.

PAR-1 is expressed in numerous tumor cell lines and its activation may play a role in tumor adhesion *in vivo,* as it was found that thrombin and the PAR-1 agonist peptide administered to tumor cell lines increased their adhesion to platelets, von Willebrand factor and endothelial cells *in vitro* (Nierodzik *et al.*, 1992; Klepfish *et al.*, 1993; Nierodzik *et al.*, 1996). It was demonstrated that PAR-1 activation is required and is rate-limiting for thrombin-enhanced experimental pulmonary metastasis (Nierodzik *et al.*, 1998) and promotes the invasion of metastatic breast carcinoma cells, in which it is preferentially expressed, in culture (Even-Ram *et al.*, 1998; Henrikson *et al.*, 1999). Thus tools for inhibiting PAR-1 activation may be useful in controlling metastasis.

Nervous system

PAR-1 is expressed in neurons and glial cells in the brain and its distribution in some areas of the brain was found to co-localise with that of prothrombin (Niclou *et al.*, 1994; Weinstein *et al.*, 1995). Treatment with thrombin or the PAR-1 agonist peptide was found to cause neurite retraction in neurons (Suidan *et al.*, 1992; Gurwitz *et al.*, 1988) and changes in the morphology of astrocytes (Beecher *et al.*, 1994; Cavanaugh *et al.*, 1990). PAR-1 activation protected both astrocytes and neurons in culture from cell death induced by hypoglycemia and oxidative stress (Vaughan *et al.*, 1995). Furthermore, thrombin and PAR-1 activating peptide treatment were found to attenuate neuronal cell death induced by β-amyloid, a protein implicated in Alzheimer's disease (Smith-Swintosky *et al.*, 1995; Pike *et al.*, 1996). Interestingly, protease nexin-1, a serine protease inhibitor which is co-expressed with prothrombin in certain regions of the brain, inhibited the effects of thrombin in neurons and astocytes (Cavanaugh *et al.*, 1990; Beecher *et al.*, 1994). It can be postulated that, following neural injury, thrombin may enter the brain and PAR-1 activation may play a protective role.

Embryogenesis

PAR-1 may be important in embryogenesis, as deletion of PAR-1 gene in mice resulted in 50% mortality at embryonic days 9.5 and 10.5 (Connoly *et al.*, 1996; Darrow *et al.*, 1996) whereas the rest of the animals become grossly normal. PAR-1 is not expressed in mouse platelets yet histological examination of the dead embryos "revealed embryonic blood cells in in the pericardial, amniotic and exocoelemic cavities suggesting a defect in hemostatic mechanisms or vascular integrity" (Coughlin, 1999).

1.1.4.b PAR-2

PAR-2 is expressed in a number of tissues, including the gastrointestinal and respiratory tracts, pancreas, kidney, liver, prostate, ovary, blood vessels, eye, bone and skin, where expression is primarily in the epithelial, endothelial and smooth muscle cells (Nystedt *et al.*, 1994; Nystedt *et al.*, 1995; Bohm *et al.*, 1996b; D'Andrea *et al.*, 1998, Abraham et al., 2000).

The precise physiological role of PAR-2 has not been elucidated as the physiological activators of PAR-2 in most tissues have not yet been identified. PAR-2 is highly expressed in the gastointestinal tract and PAR-2 activation induced contraction of gastric smooth muscle (Al-Ani *et al.*, 1995; Saffeddine *et al.*, 1996). In the gastrointestinal tract, trypsin has been suggested as an activator under physiological conditions, as trypsin activated PAR-2 in intestinal epithelial cells and induced secretion of prostaglandin E_2 and $F_1\alpha$ in enterocytes and of amylase in pancreatic acini (Bohm *et al.*, 1996; Kong *et al.*, 1997; Dery *et al.*, 1998). Tryptase may be important in pathological conditions involving mast cell infiltration and/or degranulation (Molino *et al.*, 1997b; Kawabata *et al.*, 1998). During inflammation, mast cells normally present in the colon (Aldenborg and Enerback, 1994), release tryptase, which was shown to activate PAR-2 in rat colonic myocytes (Corvera *et al.*, 1997).

Inflammation

Expression of PAR-2 in human endothelial cells is elevated by inflammatory cytokines such as tumor necrosis factor-α, interleukin-1 and bacterial lipopolysaccharide (Nystedt *et al.*, 1996), suggesting a possible role for PAR-2 in inflammatory conditions. In addition, neutrophils which are involved in the inflammatory process express PAR-2 and its

stimulation leads to neutrophil activation *in vitro* [expression of the activation molecule CD11b and shape changes] (Howells *et al.,* 1997). PAR-2 agonist peptides induce leukocyte rolling and adhesion *in vivo* (Vergnolle, 1999) and PAR-2 deficient mice exhibited a defect in leukocyte rolling and therefore delayed onset of inflammation (Lindner *et al.,* 2000).

PAR-2 activation in vascular endothelial cells induced release of von Willebrand factor (Storck *et al.,* 1996; Langer *et al.,* 1999) and expression of tissue factor, along with a decrease in coagulation time in a standard clotting assay (Alm *et al.,* 1999; Langer *et al.,* 1999), thus PAR-2 may promote platelet adhesion to the endothelium and blood coagulation. PAR-2 stimulation also increased vascular permeability (Kawabata *et al.,* 1998), which may promote thrombin production thereby triggering platelet aggregation and thus playing a role in vascular haemostasis.

PAR-2 may play a role in vascular tone, as PAR-2 activation induced endothelium-dependent relaxation of blood vessels (Al-Ani *et al.,* 1995; Saiffeddine *et al.,* 1996; Hwa *et al.,* 1996; Emilsson *et al.,* 1997; Hollenberg *et al.,* 1996b), although high concentrations of PAR-2 agonist peptide were reported to cause endothelium-dependent contraction of artery ring preparations (Roy *et al.,* 1998). In rats or mice in which the PAR-1 gene has been deleted, intravenous injection of PAR-2 agonist peptide caused a substantial decrease in blood pressure (Emilsson *et al.,* 1997; Damiano *et al.,* 1999). PAR-2 activation was found to cause relaxation of airway preparations from mouse, rat, guinea-pig and humans by release of prostaglandin E_2 (PGE_2) from the epithelium [Cocks *et al.,* 1999]. Similarly, in anasthetised rats PAR-2 activation resulted in prolonged inhibition of bronchoconstriction suggesting a protective role of PAR-2 in the airways in respiratory diseases (Cocks *et al.,* 1999).

PAR-2 is expressed in skin (Santulli *et al.*, 1995; D'Andrea *et al.*, 1998) and PAR-2 activation in human keratinocytes induces expression of the proinflammatory cytokines interleukin-6, interleukin-8 and macrophage colony stimulating factor (Wakita *et al.*, 1997; Hou *et al.*, 1998a) and was found to up-regulate keratinocyte phagocytosis (Sharlow *et al.*, 2000). PAR-2 activation on keratinocytes regulates pigmentation via keratinocyte-melanocyte interactions (Seiberg *et al.*, 2000). Tryptase has also been shown to activate PAR-2 in human keratinocytes (Schechter *et al.*, 1998), thus it has been postulated that, in inflammatory conditions, tryptase secreted from mast cells activates PAR-2, contributing to the inflammatory process.

It was recently shown that PAR-2 was activated by coagulation factors Xa and VIIa (Camerer *et al.*, 2000). Factor Xa directly activated PAR-2, while factor VIIa, after binding to tissue factor, converts inactive zymogen factor X to the active protease factor Xa, which then activates PAR-2. Factor Xa is short lived so it is possible that *in vivo*, factor VIIa and tissue factor-mediated PAR-2 activation occurs. Keratinocytes naturally express tissue factor (Camerer *et al.*, 2000) and factor VIIa was shown to activate PAR-2 in keratinocytes at concentrations that are present in circulation *in vivo*. Thus in cases of injury or inflammation, plasma coagulation proteins may come in to contact with keratinocytes to activate PAR-2. Endothelial cells can be induced to express tissue factor by inflammatory cytokines (Bevilacqua *et al.*, 1986) or following PAR-2 activation (Alm *et al.*, 1999; Langer *et al.*, 1999). Thus during vascular injury or inflammation, PAR-2 may be activated by coagulation factors and induce the expression of von Willebrand factor (Storck *et al.*, 1996; Langer *et al.*, 1999), thus promoting platelet adhesion and the previously described leukocyte adhesion and leukocyte rolling on the vascular endothelium

(Vergnolle, 1999). Thus, similar to PAR-1, PAR-2 could be involved in coagulation and inflammation processes (Fig. 1.4).

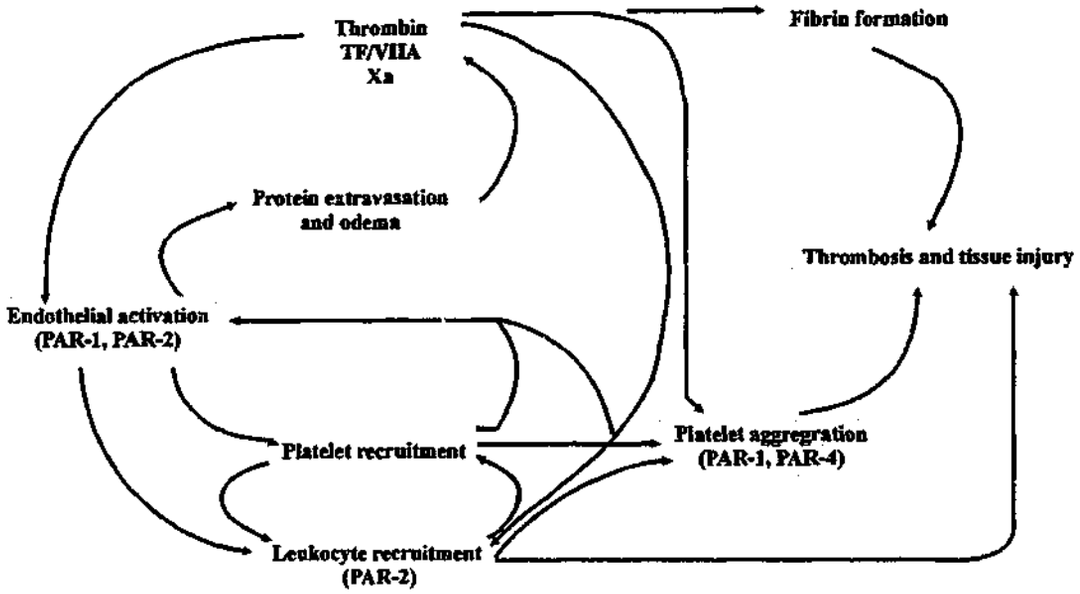


***Figure 1.4* An illustration of potential roles of PARs in coagulation and inflammatory process.** Activation of PARs on endothelial cells, leukocytes and platelets by coagulation proteases may possibly provide a link between coagulation and inflammation. (Adapted frrom Coughlin, 2000)

Mitogenesis

Activation of PAR-2 has been found to have different effects on proliferation in various cell types. Activation of the receptor inhibits keratinocyte cell growth (Derian *et al.*, 1997) and colony formation in a lung adenocarcinoma cell line (Böhm *et al.*, 1996). However, PAR-2 activation mediates mitogenesis in endothelial cells (Mirza *et al.*, 1996), murine lymphoid cells (Mirza *et al.*, 1997), coronary artery smooth muscle cells (Bretschneider *et al.*, 1999; Bono *et al.*, 1997) and human gastric carcinoma cells, as well as stimulating

adhesion (Miyata *et al.*, 2000). Tryptase and the PAR-2 agonist peptide were shown to induce human lung fibroblast proliferation (Akers *et al.*, 2000), which may be important as many lung diseases are associated with an increased number of fibroblasts and mast cells in close proximity to fibroblasts.

Nervous system

PAR-2 was localized to astrocytes and neurons in various regions of the brain (D'Andrea *et al.*, 1998), and is expressed in primary culture of neurons and astrocytes of rat brain (Smith-Switosky *et al.*, 1997) and C6 glioma cells (Ubl *et al.*, 1998). It was found that high concentrations of PAR-2 activating peptide resulted in cell death of neurons and astrocytes (Smith-Switosky *et al.*, 1997). PAR-2 activation caused nitric oxide-dependent relaxation in the basilar artery of rat brain (Sobey and Cocks, 1998), which increased blood flow and may promote brain eodema (Sawada *et al.*, 2000). Although a physiological protease agonist of PAR-2 in brain has not been found, it was recently reported that an unidentified serine-like protease was produced following rat brain injury which was able to activate PAR-2 on human glioblastoma cells (Sawada *et al.*, 2000). It has been suggested that PAR-2 may play a role in neurogenic processes, as its activation causes the release of proinflammatory calcitonin-gene related peptide and substance P (Steinhoff *et al.*, 2000). Since PAR-2 can act as a sensor of coagulation proteases such as factors Xa and VIIa it may play a role in neurogenic inflammation following tissue injury (Camerer *et al.*, 2000).

1.1.4.c PAR-3

PAR-3 transcripts were expressed prominently in megakaryocytes in mouse spleen and bone marrow but were also detected in lung and brain (Ishihara *et al.*, 1997; Schmidt *et al.*, 1998). Although there have been reports of PAR-3 expression in human platelets (Schmidt *et al.*, 1998; Scase *et al.*, 1997), a detailed study using reverse transcription PCR, Northern blot analysis and FACS analysis failed to detect PAR-3 expression on human platelets (Kahn *et al.*, 1999). PAR-3 was found to be expressed on human umbilical vein endothelial cells (Schmidt *et al.*, 1998) and it has been suggested that it may play a role in megakaryocyte or endothelial cell development.

PAR-3 is expressed in mouse platelets (Ishihara *et al.*, 1997) and it was found that platelets from a PAR-3 null mouse responded to high but not low concentrations of thrombin (Kahn *et al.*, 1998a). The responsiveness to high thrombin concentrations was ascribed to PAR-4 signalling, which is also expressed on mouse platelets (Kahn *et al.*, 1998a). COS cells or *Xenopus* oocytes expressing human PAR-3 cDNA responded to low concentrations of thrombin, inducing phosphoinisitol hydrolysis (Ishihara *et al.*, 1998). Interestingly, cells expressing mouse PAR-3 cDNA did not respond to thrombin (Nakanishi-Matshui *et al.*, 2000). Co-expression of mouse PAR-3 with mouse PAR-4 resulted in cleavage of PAR-4 and mediated signalling to low concentrations of thrombin, compared to PAR-4 expression on its own. The evidence strongly suggested that the N-terminal extracellular domain of PAR-3 and in particular the thrombin-interacting sequences were necessary for this process (Nakanishi-Matshui *et al.*, 2000) and thus it appears that mouse PAR-3 acts as a cofactor, enhancing the cleavage and activation of mouse PAR-4.

1.1.4.d PAR-4

PAR-4 is expressed in several tissues, with high levels in lung, pancreas, thyroid, testis and small intestine (Xu *et al.*, 1998). PAR-4 is expressed in human platelets and induces platelet activation as shown by the finding that the PAR-4 agonist peptide alone was sufficient to trigger platelet aggregation and secretion (Kahn *et al.*, 1998a; Kahn *et al.*, 1999). Pre-incubation of human platelets with blocking antibodies to the thrombin interaction site in PAR-1 inhibited platelet activation at low but not high concentrations of thrombin (Kahn *et al.*, 1999; Hung *et al.*, 1992a; Brass *et al.*, 1992). PAR-4 blocking antibodies did not prevent the thrombin-induced platelet activation but pre-treatment of platelets with both PAR-1 and PAR-4 antibodies blocked platelet aggregation even at high concentrations of thrombin (Kahn *et al.*, 1999). Thus it has been suggested that PAR-4 mediates activation of human platelets by high concentrations of thrombin (Coughlin, 2000).

1.1.5 Identification of New Proteases Capable of Activating PARs

PARs can be activated by any protease that can cleave the N-terminus at the unique site required for exposure of the tethered ligand. The entire spectrum of proteases that activate PARs and induce intracellular signals has not yet been elucidated (Coughlin, 2000). Since the only requirement for PAR activation is proteolysis at the cleavage site of the receptor and a number of proteases have been found to activate the same receptor, it is indeed possible that PARs can be activated by other proteases yet to be determined.

Identification of other proteases capable of activating PARs would allow us to further understand the role of these receptors in physiology and disease. PARs might confer responsiveness not only to endogenous but also exogenous proteases as long as these "foreign" proteases have specificity for the cleavage site of the receptors. Potential roles of PARs have been identified in disease, linking coagulation and inflammatory responses (Couglin, 2000). An example of inflammatory disease state in which human cells are exposed to exogenous proteases is in bacterial infections, where the pathogens produce powerful proteolytic enzymes that can affect the host's tissues (Travis *et al.*, 2000). One such disease is periodontal disease. Three members of the PAR family, namely PAR-1, -2 and -4 are activated following cleavage after an arginine residue in their extracellular domain and therefore a bacterial protease that could specifically cleave after an arginine residue would have the potential to activate these receptors on the surface of human cells.

1.2 Contribution of proteolytic enzymes to bacterial pathogenesis

Bacterial pathogens have a range of virulence factors, which facilitate colonisation and evasion of host defence and promote damage to the host's tissue during infection. Common virulence factors that these pathogens produce are proteolytic enzymes (proteases). Their major role is thought to be degradation of the host's tissues and thus provision of the nutrients necessary for growth and proliferation of the bacteria (Payne, 1976). However, recent findings have shown that bacterial proteases can also disrupt a number of the host's systems and thus help the bacteria to survive in the host (Travis *et al.*, 1995; Maeda and Yamamoto, 1996). The bacterium *Porphyromonas gingivalis*, a major etiological agent in periodontal diseases, produces proteases called gingipains that can affect cascade systems, deregulate cytokine networks and cleave cell surface receptors.

1.3 Periodontal Disease

Periodontal disease is a term used to describe a number of conditions that afflict the periodontium (gingiva, periodontal ligament, cementum, alveolar process and junctional epithelium). Two common conditions are gingivitis and periodontitis. Chronic marginal gingivitis is characterised by gingival inflammation and redness, oedoma, bleeding, changes in contour of the gingiva and increased flow of gingival crevicular fluid (a serum exudate). "Periodontitis is clinically differentiated from gingivitis by the loss of connective tissue attachment to the teeth in the presence of concurrent gingival inflammation, loss of periodontal ligament and disruption of its attachment to the cementum, gum regression, tooth loss and alveolar bone resorption" (Schenkein, 1999) [Fig. 1.5].

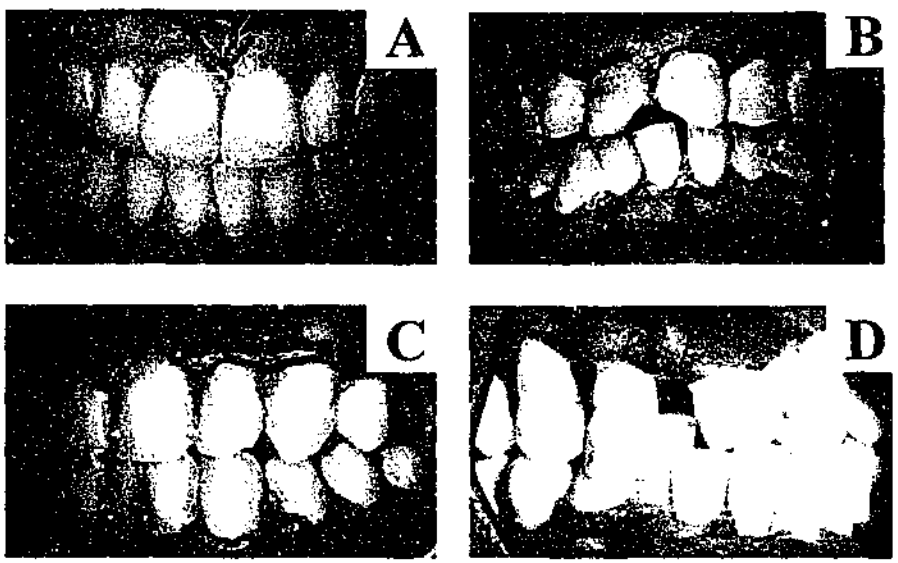


Figure 1.5. **Progression of periodontal disease.**
Appearance of human gums and teeth in individuals showing A) no periodontal disease; B) early gingivitis; C) moderate gingivitis and D) periodontitis.

Cytokines are molecules that are released by host cells into the local environment, where they act as molecular signals for other cells and induce specific responses. A number of pro-inflammatory cytokines such as IL-1, IL-6, IL-8, TNF-α and pro-inflammatory mediators such as prostaglandin E_2 (PGE_2) are found in gingival tissues and gingival crevicular fluid from patients with periodontal disease (Schenkein, 1999). These pro-inflammatory cytokines, together with bacterial components and activation of the host's complement and kinin systems, most likely promote the observed massive infiltration of neutrophils into the gingival tissue (Attstom *et al.*, 1970; Hellden *et al.*, 1973; Schenkein, 1999; Page, 1998). It has been suggested that neutrophils, which should provide protection against bacteria, can possibly contribute to local gingival injury. Neutrophil death and/or extracellular degranulation, whereby cytoplasmic granular enzymes of neutrophils are released into the extracellular space, can have negative effects on the surrounding tissue (Schenkein, 1999; Page, 1998; Travis and Potempa, 2000).

Enzymes such as the connective tissue degrading proteases, elastase, cathepsin G and protease 3, are capable of degrading collagen and basement membrane components and thus contribute to destruction of the host's tissues. In addition, the pro-inflammatory cytokines induce the production of PGE_2 and matrix metalloproteases (MMP), which are destructive to the extracellular matrix of the gingiva and the periodontal ligament and promote resorption of the alveolar bone (Birkedal-Hansen, 1993; Offenbacher, 1996; Schenkein, 1999; Page, 1998).

1.3.1 Microbial Etiology

Initiation and progression of periodontal disease depends on the presence of pathogenic bacteria. Oral surfaces are coated with a pellicle, which consists mainly of glycoproteins from saliva. These molecules are absorbed on the tooth surface and serve as substrates for bacterial adherence. Gram-positive bacteria from saliva express adhesins that bind to the pellicle, mediate colonisation and initiate the formation of plaque. The plaque, if left undisturbed for two days, is colonised by Gram-negative bacteria through receptors that bind to the Gram-positive bacteria (Page, 1998; Lamont *et al.*, 1993; Nyvad and Kilian 1987; Loe *et al.*, 1969).

Plaque bacteria bind to the tooth surface and extend into the gingival sulcus and subsequently form subgingival plaques into the periodontal pocket. The junction between the tooth and the gingiva is termed the gingival cuff and is comprised of two zones. Coronally lies the sulcular epithelium, which is separated from the enamel of the tooth by a small space known as the gingival sulcus. At the base of the gingival sulcus, the sulcular epithelium merges with the junctional epithelium. The junctional epithelium forms a thin,

epithelial lining directly attached to the tooth. The periodontal pocket is an abnormal deepening and enlargement of the gingival sulcus that occurs when the plaque bacteria colonise and disrupt the attachment between the junctional epithelium and the tooth, as periodontal disease progresses (Fig. 1.6).

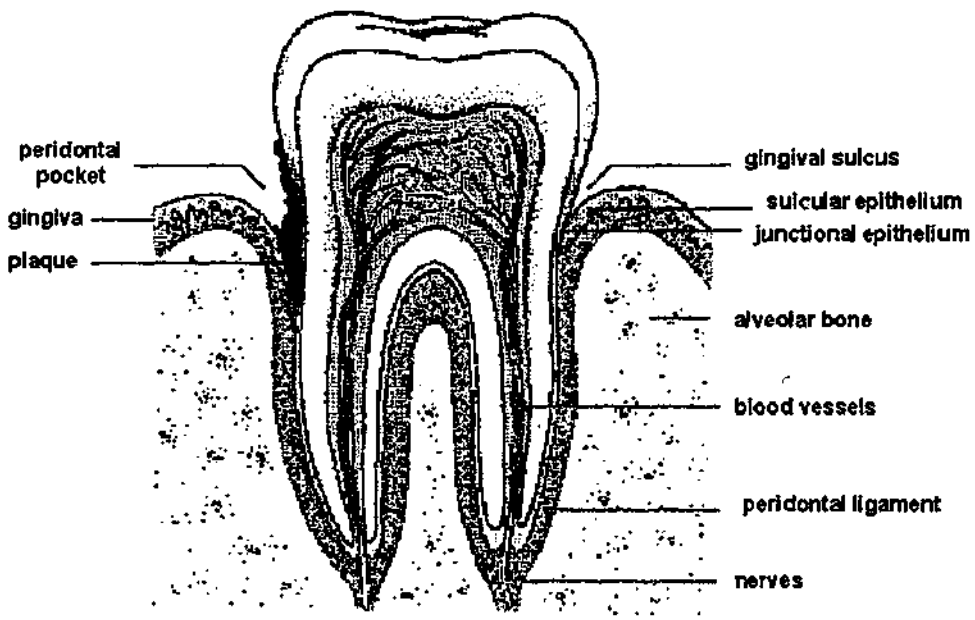


Figure 1.6. **Longitudinal section of gingiva and tooth.**
Plaque bacteria colonise and disrupt the attachment between the junctional epithelium and the tooth causing deepening and enlargement of the gingival sulcus known as a periodontal pocket.

These bacterial plaques have the characteristics of biofilms (Darveu *et al.*, 1997). Biofilms are defined as "matrix-enclosed bacterial populations adherent to each other and/or to surfaces or interfaces" (Costerton *et al.*, 1994). A biofilm is an ecological community where there is metabolic cooperativity between the bacteria, forming a complex structure that seems to be a primitive circulatory system. "Within the biofilm there are

numerous microenvironments with different pH, oxygen concentrations and electric potentials. The biofilm surface appears to be resistant to the host's defences and to antibiotics" (Costerton *et al.*, 1994). Although 300 species of microorganisms have been isolated from dental plaque, only a small percentage of these are etiologic agents for periodontal disease (Moore *et al.*, 1994). Three species of Gram-negative anaerobic or facultative bacteria account for most cases of periodontitis: *Actinobacillus actinomycetemcomitans, Bacterioides intermedius* and *Porphyromonas gingivalis* (Newman, 1996).

1.4 *Porphyromonas gingivalis*

P. gingivalis is identified as a major etiologic agent in the periodontal disease process in humans (Slots *et al.*, 1986; Haffajee and Socransky, 1994; Socransky and Haffajee, 1992; Newman, 1996) and has been recognised as a virulence agent that initiates progression of periodontitis in primate and rodent models of periodontal destruction (Holt *et al.*, 1988). *P. gingivalis* is a gram-negative, anaerobic, non-motile, non-sporing, asaccharolytic bacterium (Fig. 1.7) that depends on nitrogenous substrates for energy (Shah *et al.*, 1989). It is limited in its ability to ferment free amino acids, yet it can efficiently use peptides for growth (Shah *et al.*, 1989). This bacterium also requires iron for growth and in order to satisfy this nutritional need it utilises hemin (iron protoporphyrin IX) (Barua *et al.*, 1990; Smalley *et al.*, 1996; Bramanti *et al.*, 1991; Fujimura *et al.*, 1996). Hemoglobin, haptoglobin, myoglobin, hemopexin, methemoglobin, oxyhemoglobin, albumin and lactoperoxidase are some of the hemin-containing molecules that the bacterium can utilise following proteolytic processing (Barua *et al.*, 1990; Smalley, *et al.*, 1996; Bramanti *et al.*,

1991; Fujimura *et al.*, 1996). Hemin is stored on the surface of *P. gingivalis* cells, probably accounting for its characteristic black-pigmented appearance (Genco, 1995).

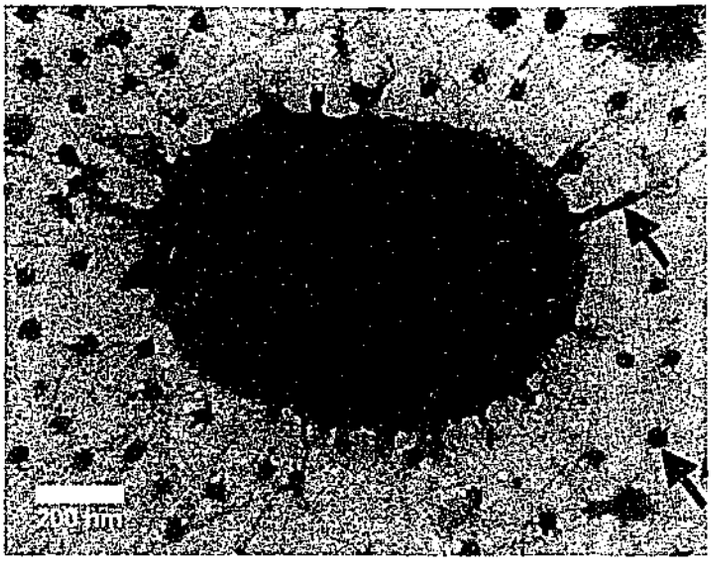


Figure 1.7. **An electron micrograph of a *Porphyromonas gingivalis* cell.** Membrane fragments and membrane vesicles derived from the outer membrane of the bacterium are seen here (arrows).

P. gingivalis is equipped with a number of virulence factors that enable it to colonise and survive in the gingival sulcus or the periodontal pocket and contribute to the development of disease. Such virulence factors are lipopolysaccharide, fimbriae, haemagglutinins and proteases. Proteolytic enzymes, termed gingipains, have been the focus of research over the last ten years and are thought to play an intrinsic role in the pathogenicity of *P. gingivalis*.

1.4.1 Lipopolysaccharide

Lipopolysaccharide (LPS) produced by *P. gingivalis* can activate osteoclasts directly and induce release of PGE_2 and the cytokines IL-1β, TNF-α and IL-6 from macrophages, monocytes and fibroblasts (Bramanti *et al.*, 1989; Lindeman *et al.*, 1988; Roberts *et al.*,

1997; Sismey-Durrant *et al.*, 1991; Yamaji *et al.*, 1995; Koka *et al.*, 1997). IL-1β, TNF-α and IL–6 are pro-inflammatory cytokines that contribute to the inflammation observed in periodontal diseases and the influx of inflammatory cells. In addition, they are also potent local mediators of bone resorption, and can inhibit collagen synthesis by osteoblasts and induce the production of host metalloproteases that destroy connective tissue and bone (Havemosse-Poulsen *et al.*, 1997; Holt *et al.*, 1991). *P. gingivalis* LPS has also been found to stimulate bone resorption *in vitro* (Miyata *et al.*, 1997).

1.4.2 Fimbriae

Ultrastructural examination has revealed the presence of peritrichious fimbriae, 0.3 to 3.0 μm long and 5 nm wide, on most strains of *P. gingivalis* (Okuda *et al.*, 1981). The major type of fimbriae consists of a fimbrillin monomer subunit (Lee *et al.*, 1991). Fimbriae probably have a central role in establishing bacterial adherence to saliva coated surfaces in the oral cavity (Amano *et al.*, 1996; Amano *et al.*, 1998), fibronectin (Muramaki *et al.*, 1996), lactoferrin (Sojar *et al.*, 1998), other bacteria (Goulbourne and Ellen, 1991; Lamont *et al.*, 1993) and to epithelial cells (Isogai *et al.*, 1988). Fimbriae also appear to play an important role in infection, as immunisation with purified fimbriae resulted in protection against periodontal destruction in a gnotobiotic rat model (Evans *et al.*, 1992). In addition, inactivation of the gene encoding fimbrillin (*fim*A), resulted in a mutant strain that did not produce fimbriae and had significantly reduced ability to cause periodontal bone loss in a gnotobiotic rat model (Malek *et al.*, 1994). *P. gingivalis* fimbriae stimulated bone resorption *in vitro* (Kawata *et al.*, 1994), by inducing the expression of IL-1β in bone cells (Kawata *et al.*, 1994), and also up-regulating the expression of IL-1β, TNF-α and IL-6

from monocytes and macrophages and IL-1β and TNF-α production from gingival fibroblasts (Hanazawa *et al.*, 1988; 1991; Ogawa *et al.*, 1991; 1994a; 1994b). The production of pro-inflammatory cytokines (IL-1β, TNF-α and IL-6) probably promotes the inflammatory state that describes periodontal disease.

1.4.3 Haemagglutinins/Adhesins

To date five haemagglutinin genes (*hagA, B, C, D, E*) have been cloned from *P. gingivalis* and encode distinct molecules (Lamont *et al.*, 1998). *P. gingivalis* uses heme for growth, thus adherence of bacterial cells to erythrocytes may also serve a nutritional function (Lepine *et al.*, 1996). When expressed on the bacterial cell surface, haemagglutinins may facilitate bacterial colonisation by binding to receptors on human cells (Lamont *et al.*, 1998). The role of haemagglutinins in adhesion to host cells is supported by the fact that two of the *hag* genes were identified through screening of *E. coli* clones expressing *P. gingivalis* genes that mediated attachment to epithelial cells (Duncan *et al.*, 1996).

Researchers had observed an association between the haemagglutinating and proteolytic activity of *P. gingivalis* (Hoover *et al.*, 1992; Nishikata and Yoshimura, 1991). It has been found that haemagglutinin-related sequences are present not only as independently expressed genes, e.g., *hagA*, but also on genes encoding proteolytic proteins termed gingipains R and K, e.g. ArgI from strain W50 of *P. gingivalis* (Pavloff *et al.*, 1995; Aduse-Opoku *et al.*, 1995; Okamoto *et al.*, 1995). A sequence within the haemagglutinin/adhesion domain of ArgI is also present four times in the HagA protein (Han *et al.*, 1996) and once in the TonB-dependent protein (Aduse-Opoku *et al.*, 1997). A monoclonal antibody that reacts with this common sequence was found to inhibit heamagglutination of *P. gingivalis* (Curtis *et al.*, 1996).

1.5 Gingipains

Proteases produced by *P. gingivalis* were found to cleave C-terminal to arginine (Arg-Xaa) or lysine (Lys-Xaa) residues within proteins or peptides and due to their substrate specificity were initially termed "trypsin-like". Large amounts of these proteases are bound to the cell surface of whole bacteria, membranous fragments and vesicles - which are blebs of the outer membranes- and also in a soluble form released into the medium. These proteases are cysteine proteases, with Arg-Xaa and Lys-Xaa specificity, termed gingipains-R and -K respectively, and account for up to 85% of the entire proteolytic activity of the bacterium (Kuramitsu, 1998; Potempa and Travis, 1998).

The first gene encoding a gingipain to be identified was denoted *rgp1* (arg gingipain-1) from strain H66 (Pavloff *et al.*, 1995), followed by *prpR1* (protease polyprotein ArgI) from strain W50 (Opoku *et al.*, 1995), and *rgpA* (argingipain A) from strain 381 (Okamoto *et al.*, 1995). Sequence analysis revealed that these loci are related and represent homologous genes and proteins in different strains. Translation of these *rgp1*-like genes and sequence analysis revealed an N-terminal pro-peptide domain adjacent to a 50kDa catalytic domain, followed by a C-terminal extension that contained sequences previously found in haemagglutinins of *P. gingivalis*. The existence of a second arginine-specific protease gene was demonstrated by Nakayama *et al.* (1995) in strain ATCC 33277 termed *rgpB*, followed by the identification of *rgp-2* in strain H66, *prtRII* and *prR2* in strain W50 (Mikolajczyk-Pawlinska *et al.*, 1998; Slakeski *et al.*, 1998; Rangarajan *et al.*, 1997b). In strain W50, this second gene exhibits 80% similarity and 77.5% identity to the first gene, as it contains the pro-peptide and catalytic domain, yet lacks the C-terminal extension (736 vs 1706aa) (Rangarajan *et al.*, 1997b). As with the first arginine-specific

protease gene, the second locus described in the different strains represents homologous genes. Similarly, genes whose products exhibited lysine protease activity were identified in various strains of *P. gingivalis*: *prtP*, (W12 strain), *kgp* (H66 and 381), *kgp*(381)-*hagD*, (381), *prtP* (W83), *prtK* (W50) (Barkocy-Gallagher *et al.*, 1996; Lewis *et al.*, 1998; Pavloff *et al.*, 1997; Okamoto *et al.*, 1996; Slakeski *et al.*, 1998). Translation of these genes revealed that they were highly homologous and contained a catalytic and haemagglutinin domain.

In order to overcome the redundancy and confusion arising from the initial nomenclature of the genes and their products, Curtis *et al.* (1999) have proposed a unified nomenclature for the gingipain genes and their proteins: Gingipains-R (Rgps) occur in either soluble (RgpA, HRgpA, RgpB) or membrane-associated forms (mtRgpA, mtRgpB) and are products of two related genes, *rgpA* and *rgpB* (Curtis *et al.*, 1999). The *rgpA* gene-encoded soluble gingipains are released into the medium as the single chain 50kDa RgpA protease (Chen *et al.*, 1992) or the high-molecular weight HRgpA, which is 95kDa and consists of the 50kDa catalytic domain (RgpA) non-covalently complexed with haemagglutinin/adhesin subunits (Pike *et al.*, 1994; Rangarajan *et al.*, 1997a). The related *rgpB* gene lacks the coding region for the haemagglutinin domain and codes for a 507 amino acid residue protein (Mikolajczyk-Pawlinska *et al.*, 1998). The mature C-terminally truncated product of *rgpB* termed RgpB, is a single chain 48kDa protein essentially identical to RgpA, although the two proteins differ considerably within the C-terminal region after residue 363 (Potempa *et al.*, 1998). The *rgpA* gene also encodes mtRgpA (mt= membrane type), which is the highly post-translationally lipopolysaccharide modified 70-80kDa membrane-associated form of RgpA (Rangarajan *et al.*, 1997a; Travis and Potempa, 2000). Similarly, the *rgpB* gene also encodes for mtRgpB, which is the membrane-

associated form of RgpB (Rangarajan *et al.*, 1997b) [Figure 1.8]. The gene for the lysine-specific protease activity is termed *kgp*. There is less information regarding the maturation of *kgp*-derived enzymes, yet a high molecular weight form analogous to HRgpA has been purified (Pike *et al.*, 1994). It was termed Kgp and consists of a pro-peptide and catalytic domain along with a C-terminal extension, which is highly homologous to the haemagglutinin domain of HRgpA.

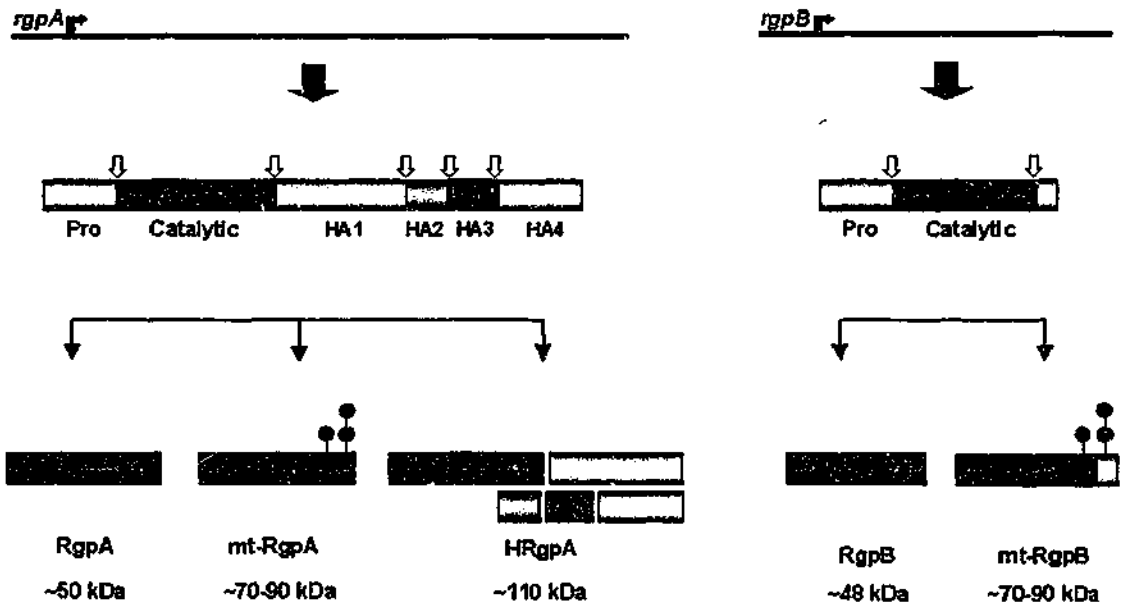


Figure 1.8. **Schematic representation of the translation of the different isoforms of gingipains-R.** Translation of *rgpA* and *rgpB* genes produces membrane forms (mt-RgpA, mt-HRgpA and mt-RgpB) and secreted forms (HRgpA, RgpA and RgpB) of the enzymes. HA represent different adhesin domains. (Adapted from Curtis *et al.*, 1999).

Rgps have an exclusive specificity for cleavage after arginine residues and are thiol dependent and thus require activation by reducing agents such as cysteine (Potempa *et al.*, 1995b). Alkylation studies have identified Cys244 as the putative reactive-site residue (Nishikata and Yoshimura, 1995; Potempa *et al.*, 1998). Rgps are inhibited by the bacterially-derived peptidyl aldehyde inhibitors, leupeptin and antipain (Potempa *et al.*, 1995b) and by thiol group blocking agents such as iodoacetamide and iodoacetate (Potempa *et al.*, 1995b). Their activity is also blocked not only by Arg-chloromethoketone inhibitors, but also by Lys-chloromethoketone inhibitors or other residues at P1 acting as irreversible covalently attached inhibitors (Nakayama, 1997; Potempa *et al.*, 1997). The Rgps are stabilised by calcium (Chen *et al.*, 1992) and are inactivated by EDTA (Potempa *et al.*, 1995b; Fujimura *et al.*, 1998).

RgpB and HRgpA were purified from strain H66 with a yield of 2-6 mg of each protein from 1 L of culture medium, whereas RgpA was obtained in microgram quantities (Potempa *et al.*, 1995b). When subjected to anion exchange chromatogarphy, RgpA and RgpB behave differently; RgpA appears as a small unbound fraction whereas RgpB is bound and contains 95% of the total activity (Potempa *et al.*, 1998). Potempa *et al.*, (1998) have shown that due to truncation of its C-terminus RgpB exists in four isoforms that differ in molecular mass, isoelectric point and their affinity for arginine-Sepharose. Yet, the RgpB isoforms are "indistinguishable with regard to stability, pH optima, kinetic characteristics and proteolytic activity" (Potempa *et al.*, 1998).

1.5.1 Role of Gingipains in Virulence of P. gingivalis and Development of Periodontal Disease

The role of gingipains *in vivo* has been a topic of intense interest as gingipains have been detected in gingival crevicular fluid (plasma-like exudate) collected from periodontitis sites (Wikstrom *et al.*, 1994), and anti-gingipain antibodies are present in serum from patients with periodontal disease (Kelly *et al.*, 1994; Genco *et al.*, 1999). In a murine model, the virulence of *Porphyromonas gingivalis* was reduced if the bacteria used for inoculation had been treated with protease inhibitors (Kesavalu *et al.*, 1996; Genco *et al.*, 1999; Feuille *et al.*, 1996). In addition, immunisation with gingipains or peptides corresponding to the N-terminal sequence of the catalytic domain of gingipains R induced a protective immune response against *P. gingivalis* infection in a mouse chamber model (Genco *et al.*, 1998).

The creation of mutant strains of *P. gingivalis* has provided evidence that gingipains are major pathogenic factors of the bacterium. An *rgpA*-deficient mutant of P. *gingivalis* W83 had decreased Arg-X proteolytic activity and exhibited reduced virulence in a mouse model of infection (Fletcher *et al.*, 1995). A double-knockout *rgpA/rgpB* mutant of *P. gingivalis* ATCC 33277 had lost all Arg-X protease activity and exhibited decreased ability to inhibit leukocyte bactericidal function, along with reduced heamagglutinin activity (Nakayama *et al.*, 1995). Inactivation of the *kgp* gene resulted in a *P. gingivalis* strain that exhibited a reduction of virulence as tested in a mouse model examining soft tissue destruction (Lewis *et al.*, 1998). Gingipains also play a central role in nutrition, as a rgpA/rgpB/kgp triple mutant had no proteolytic activity towards gelatin or bovine serum albumin, which are some of the molecules in the gingiva that are degraded by *P. gingivalis* to obtain peptides essential for growth. This triple mutant was also unable to agglutinate

erythrocytes and bind hemoglobin, which would be necessary for heme acquisition (Shi *et al.*, 1999).

Rgps also contribute to the virulence of *P. gingivalis* and the pathology of periodontal disease as major processing enzymes. The ATCC 33277 *rgpA/rgpB* double mutant strain showed decreased Kgp activity and Kgp was found to be abnormally processed, indicating that Rgps are necessary for the processing of the Kgp precusor protein (Tomoko *et al.*, 1998). The proteolytic activity of gingipains has also been found to be important in the production of fimbriae. A *rgpA* deficient mutant of *P. gingivalis* 381 exhibited reduced production of the 43kDa fimbrillin subunit along with reduced transcription of the *fimA* gene (Tokuda *et al.*, 1996). This *rgpA* mutant strain also showed decreased binding to epithelial cells, gram-positive bacteria, and extracellular matrix proteases (Tokuda *et al.*, 1996). The expression of Rgps but not Kgp appears to be necessary for the production of fimbriae, as it was found that ATCC 33277 *rgpA/rgpB* double mutant *P. gingivalis* bacteria cells had very few fimbriae in comparison to *rgpA* or *rgpB* single mutants or wild-type cells, whereas the Kgp null mutant exhibited normal fimbriation (Nakayama *et al.*, 1996; Kadowaki *et al.*, 1998). Since fimbriae are involved in adherence of *P. gingivalis* to bacteria, epithelial cells and other oral substrates, gingipains appear to play an important role in colonisation by *P. gingivalis*. In addition, it has been found that passive immunisation of periodontal patients with a monoclonal antibody, recognising a sequence within the haemagglutinin domain of gingipains-R, prevented colonisation of periodontal pockets by *P. gingivalis* for up to 9 months (Booth *et al.*, 1996; Booth and Lehner, 1997).

1.5.2 Tissue destruction

Gingipains have been found to process and activate host matrix metalloproteases (MMP) including proMMP-1, proMMP-8, proMMP-9 and thus contribute to tissue destruction (DeCarlo *et al.*, 1997; Okamoto *et al.*, 1997; Fravalo *et al.*, 1996). MMPs are destructive to the extracellular matrix of the gingiva and the periodontal ligament and promote resorption of the alveolar bone (Birkedal-Hansen, 1993; Offenbacher, 1996; Schenkein, 1999; Page, 1998). Gingipains also directly degrade extracellular matrix proteins such as laminin and fibronectin (Pike *et al.*, 1996) and the receptor for fibronectin, specifically the α_5 and β_1 integrin subunits, on human gingival fibroblasts (Scragg *et al.*, 1999).

1.5.3 Degradation of Immunoglobulins

The proteolytic activity of *P. gingivalis* can compromise the host's immune system as it has been found that the gingipain proteases degraded human immunoglobulins IgG and IgM (Fishburn *et al.*, 1991; Sundqvist *et al.*, 1985). Inhibition of proteolysis of IgG by the *P. gingivalis* proteases enhanced phagocytosis of the bacterium, indicating that these proteases have a profound impact on the immune system (Cutler *et al.*, 1993).

1.5.4 Deregulation of cascade systems

A number of studies have shown that Rgps can have negative effects on a number the of the host's normal responses and cascade systems. "All cascade systems known to exist in mammalian species (complement, kallikrein/kinin, coagulation and fibrinolytic pathways) have a common feature which is the strict requirement for zymogen activation by cleavage

after specific Arg-X residues by a series of individually tailored serine proteases" (Travis and Potempa, 2000). Rgps, which cleave strictly after arginine residues have been found to deregulate these cascade systems.

1.5.4.a Kallikreinin/kinin system

Bradykinin (BK) is a peptide hormone that enhances vascular permeability resulting in plasma leakage from capillaries. BK is produced following cleavage of high or low molecular kininogens by plasma kallikrein, a serine protease. Kallikrein is released by cleavage of its zymogen precursor, pre-kallikrein, by a number of proteases (eg. plasmin, Hageman factor) (Kalpan *et al.* 1997). Rgps have been found to induce vascular permeability enhancement via activation of plasma prekallikrein and subsequent release of BK (Imamura *et al.*, 1994). In addition, Rgps in synergism with Kgp, have been found to release BK by directly cleaving high molecular weight kininogen (Imamura *et al.*, 1995a). This increase in vascular permeability may contribute to the production of gingival crevicular fluid (a plasma exudate) in periodontal disease patients. The plasma proteins found in the plasma exudate can also serve as a source of nutrients for the bacterium. Studies have shown that activation of the kallikreinin/kinin cascade by bacterial proteases can facilitate pathogen dissemination from the site of infection to the systemic circulation (Maeda and Yamamoto, 1996; Sakata *et al.*, 1996).

1.5.4.b Coagulation Cascade

Rgps have been reported to activate factor X and prothrombin resulting in the production of α-thrombin (Imamura *et al.*, 1997; Imamura *et al.*, 2000), which is the final protease of the

coagulation pathway and converts fibrinogen to a fibrin clot. Rgps also cleave protein C to release activated protein C, which inhibits coagulation (Horosaki *et al.*, 1999). Protein C activation by Rgps was also found to simultaneously decrease the concentration of protein C in plasma however, depleting the blood coagulation system of a negative regulatory mechanism (Horosaki *et al.*, 1999). The activation of protein C by Rgps in addition to activation of factor X and prothrombin may disrupt the coagulation system either locally or systemically *in vivo*. At the same time, the ability of Gingipain-K in plasma to efficiently cleave fibrinogen may influence the formation of a clot and its stability *in vivo* (Imamura *et al.*, 1995b).

1.5.4.c Complement Cascade

It has been documented that Rgps can potentially interfere with complement, which the host uses as a line of defence in order to induce bactericidal activity, opsonise bacteria and recruit phagocytic cells. Rgps efficiently cleave C5 at two specific Arg-X positions, to yield the potent chemotactic factor C5a (Wingrove *et al.*, 1992), which is known to recruit phagocytic cells. The digest products of C5 by RgpA caused enzyme release from and chemotaxis of human neutrophils and polarisation of eosinophils (Wingrove *et al.*, 1992; DiScipio *et al.*, 1996). The local production of C5a could be a mechanism for recruiting neutrophils into the inflamed tissue. However, Rgps and Kgp were also found to efficiently degrade C3 *in vitro* (Wingrove *et al.* 1992), and digestion of C3 by gingipains has been found to suppress phagocytosis of *P. gingivalis* (Cutler *et al.*, 1993; Schenkein *et al.*, 1995), which may contribute to the persistence of bacterial pathogens in the periodontal pocket.

1.5.5 Deregulation of cytokine networks

In response to infection with *P. gingivalis,* the host can produce inflammatory mediators and indeed high levels of pro-inflammatory cytokines are found in the gingiva and the gingival crevicular fluid from patients with periodontal disease (Schenkein, 1999). Rgps were found to degrade TNF-α, IL-6, IFN-γ and IL-1β *in vitro*, rendering these cytokines inactive (Calkins *et al.*, 1998; Fletcher *et al.*, 1998; Fletcher *et al.*, 1997; Banbula *et al.*, 1999). Yet interestingly, HRgpA, RgpB and Kgp cleaved IL-8 to produce an N-terminal truncated product which had three-fold higher neutrophil activating ability than the original IL-8 molecule (Mikolajczyk-Pawlinska *et al.*, 1998). In contrast, treatment of IL-8 with gingipains associated with vesicles or whole bacterial cells degraded the cytokine (Mikolajczyk-Pawlinska *et al.*, 1998). The investigators postulated that soluble gingipains may cleave IL-8 which is present in the crevicular fluid to produce the potent chemotactic truncated product that promotes neutrophil accumulation. Simultaneously, vesicle-bound gingipains, which are found close to the plaque surface, degrade IL-8 and disrupt the chemotactic gradient, thus keeping neutrophils at sites distal to the surface of the bacterial plaque.

1.5.6 Interaction of *P.gingivalis* proteases with cell surface receptors

P. gingivalis proteases can interact not only with extracellular matrix proteins and soluble proteins and enzymes but also with receptors expressed on the surface of cells. An unidentified leupeptin-insensitive protease from *P. gingivalis,* was found to stimulate neutrophils and induce superoxide anion production (Lala *et al.*, 1994). This protease was

found to cleave the formyl peptide receptor (fMLP) on the surface of neutrophils, following which the cells no longer responded to the fMLP peptide. Exposure of kidney cells to *P. gingivalis* cells caused hydrolysis of occludin, E-cadherin and β_1-integrin (Katz *et al.*, 2000), suggesting that bacterial proteases are capable of cleaving these cell surface molecules. Gingipains have been found to cleave the receptor for fibronectin on human gingival fibroblasts (Scragg *et al.*, 1999) and cause changes in cell morphology. Treatment of monocytes with gingipains caused proteolysis of CD14 (Sugawara *et al.*, 2000), which functions as a major receptor for LPS (Wright *et al.*, 1990). Kgp along with an unidentified vesicle-bound protease, but not Rgps, were found to inactivate the C5a receptor on the surface of neutrophils (Jagels *et al.*, 1996). Protease activated receptors may also be affected by gingipains as they are activated via proteolysis. Three members of the PAR family namely PAR-1, -2 and -4, are activated following cleavage after an arginine residue, thus Rgps, which are specific for arginine bonds, could potentially activate these receptors.

This thesis investigates the ability of Rgps to cleave and activate PARs expressed on the surface of human cells, and thus elicit intracellular signals leading to a cellular response. The effect of Rgps on cells that may play a role in periodontal disease was examined. In periodontal disease there is massive neutrophil accumulation into the gingiva and thus it is likely that neutrophils come in contact with *P. gingivalis* and its products. Treatment of human neutrophils, which express PAR-2, with RgpB caused calcium mobilization, and desensitization studies with trypsin and RAP indicated that RgpB activated PAR-2. The ability of RgpB to activate PAR-2 was verified by the finding that RgpB activated PAR-2 on transfected cells stably expressing the receptor.

Immunohistochemical studies have shown that *P. gingivalis* cells are in close contact with the gingival epithelium in the periodontal pockets (Noiri *et al.*, 1997),

therefore it is of interest to examine whether the bacterium's products can affect gingival epithelial cells. The KB60 cell line is an oral epidermoid cell line that has been extensively used as a model to study gingival epithelial cells. It was determined that KB60 cells express PAR-1, -2 and –3. RgpB induced calcium mobilisation in these cells and up-regulation of IL-6 expression. Calcium desensitisation studies with trypsin and thrombin in KB60 cells, in addition to the demonstrated ability of RgpB to activate PAR-1 and PAR-2 in transfected cells expressing the receptors, indicated that RgpB cleaved PAR-1 and PAR-2 in KB60 cells.

Periodontal disease has been associated with atherosclerosis, thromboembolic events and cardiovascular disease and since platelets play a central role in arterial thrombosis the effect of gingipains-R on platelets was examined. HRgpA and RgpB were found to induce an increase in intracellular calcium in human platelets and cause platelet aggregation, both of which were dependent on their proteolytic activity. Based on desensitization studies carried out with thrombin, and receptor agonist peptides HRgpA and RgpB appeared to be activating PAR-1 and PAR-4, which are expressed on the surface of platelets. This was strongly supported by the finding that HRgpA and RgpB potently activate PAR-1 and PAR-4 in transfected cells stably expressing these receptors.

CHAPTER 2

Materials and Methods

2.0 Materials

Human α-thrombin was prepared by Weiwen Dai (Dept. Biochemistry & Mol. Biology, Monash University, Australia) as previously described (Stone & Hofsteenge, 1986) and was fully active. All columns used for protein purification and Ready to Go U Prime Synthesis beads were purchased from Pharmacia (Australia). Fura-2 acetomethyl ester (Fura-2 AM) and the nuclear dye LDS-751 were purchased from Molecular Probes (Australia).

Anti-gingipain-R (RgpB) antibodies (Potempa *et al.,* 1995a) and HRgpA were provided by Dr. Jan Potempa (Department of Biochemistry, UGA, USA). Anti-PAR-1 antibodies were supplied by Dr Alison Jenkins (Department of Haematology, University of Cambridge, UK). These antibodies were raised against the peptide sequence SFLLRNPNDKYEPF corresponding to the sequence C-terminal of the cleavage site of PAR-1 (Jenkins *et al.,* 1993). Anti-bromelain antibodies (Pike *et al.,* 1997) were provided by Dr R. N. Pike (Dept. Biochemistry & Mol. Biology, Monash University, Australia).

Tissue culture media and media supplements were obtained from Trace Biosciences (Australia) and Gibco BRL (Australia). Chinese hamster ovary cells (CHO) and Chinese hamster ovary cells stably expressing human PAR-2 (CHO PAR-2) were provided by COR, San Francisco, USA. N1LF cells and N1LF stably expressing human PAR-1 (N1LF PAR-1) and N1LF stably expressing human PAR-4 (N1LF PAR-4)

(Andrade-Gordon *et al.*, 1999) were provided by Dr Patricia Andrade-Gordon, Johnson and Johnson Pharmaceuticals, USA. N1LF cells are immortalised murine lung myofibroblasts derived from PAR-1-deficient mice, which lack functional PAR-1, PAR-2, PAR-4. KB 60 cells were purchased from ATCC, USA.

ELISA kits for detection of IL-6, TNFα and INF-γ were purchased from Endogen, CSL Biosciences (Australia). Anti-mouse Immunoglobulin peroxidase conjugate and anti-mouse Immunoglobulin alkaline phosphatase conjugated was purchased from Silenus (Australia). Mouse anti-human integrin β1 monoclonal antibody was obtained from Chemicon, Silenus, (Australia). Anti-human CD11b monoclonal antibody conjugated to phycoerythrin and the nuclear dye LDS-751 was purchased from Becton Dickinson (Australia).

Protein molecular weight markers for SDS-PAGE electrophoresis Wide Range Molecular weight Standards and SeeBlue Pre-stained standards used for western blots were purchased from Novex, (Australia). DNA molecular weight markers SPP1/EcoR1 and MassRuler DNA Ladder mix were purchased from Progen (Australia) and Promega (Australia). RNA isolation reagent TRI was purchased from Sigma (Australia). The staining reagent Silver Stain Plus kit was obtained from Bio Rad (Australia).

Unless otherwise stated, all other reagents were from Sigma Chemical Co. (Australia) or ICN (Australia).

2.1 Cell Culture and Cell Handling

2.1.1 Continuous Cell Lines

Cell line	cell type	reference
KB-60	human epidermoid cancer cell line	ATCC Cell lines #CCL-17
CHO	Chinese hamster ovary cells	Smith et al., (2000)
CHO PAR-2	CHO cells stably expressing human PAR-2	Smith et al., (2000)
N1LF	immortalised murine lung myofibroblasts derived from PAR-1 deficient mice, lacking PAR-1,-2,-4	Andrade-Gordon et al., (1999)
N1LF PAR-1	N1LF cells stably expressing human PAR-1	Andrade-Gordon et al., (1999)
N1LF PAR-4	N1LF cells stably expressing human PAR-4	Andrade-Gordon et al., (1999)

2.1.2 Cell Culture

The adherent cell lines KB-60, CHO, CHO PAR-2, N1LF, N1LFPAR-1, N1LFPAR-4 were grown in a humidified atmosphere with 5% CO_2 at 37°C in the appropriate media:

KB-60 cells were grown in Eagle's medium containing Earle's balanced salts containing 5% (v/v) FCS. CHO were grown in DMEM: Ham's F12 (1:1) supplemented with 10% (v/v) FCS. The CHO PAR-2 cells were grown in the same medium as the CHO cells, which in addition was supplemented with G418 (40 units/mL). N1LF cells, were grown in DMEM containing glucose, 4 mM L-glutamine and 10% heat inactivated FCS. N1LF PAR-1, N1LF PAR-4 cells were grown in the same medium as the N1LF cells that in addition contained Hygromycin B (200 μg/mL). All growth media were supplemented with penicillin (100 U/mL) and streptomycin sulphate (100 μg/mL). Culture media were replaced every 3-4 days.

To passage KB-60, CHO and CHO PAR-2 cells, the cells were rinsed with PBS and detached by treatment of cells with trypsin-EDTA (0.05% (w/v) trypsin, 0.02% (w/v) EDTA) for 5 min at 37°C. The cells were resuspended in 10 mL of the appropriate complete culture medium and were then centrifuged at 200 × *g* for 5 min. The supernatant was removed and cells were resuspended in pre-warmed culture medium and split as required (KB-60 cells 1:5 and CHO, CHO PAR-2 cells 1:10).

N1LF, N1LF PAR-1 and N1LF PAR-4 cells were passaged by rinsing with trypsin-EDTA following which the cells were detached with trypsin-EDTA. The cells were resuspended in 10 mL of the appropriate pre-warmed complete culture medium and split 1:20.

2.1.3 Cryopreservation of cells

Cells were detached by treatment with trypsin-EDTA centrifuged as above and resuspended to a concentration of 2 x 10^6 cells/mL in a sterile freezing medium [90% (v/v) complete media, 10% (v/v) DMSO]. The cell suspension was added to cryopreservation vials and placed in a polystyrene container at – 80°C for 24 hrs. The frozen cell suspensions were then transferred to cryogenic storage containers with liquid N_2.

2.1.4 Recovery of Cryopreserved Cells

Frozen cells were removed from storage in liquid N_2 and thawed in a 37°C water bath. The cells were placed in a 15 cm^2 flask containing pre-warmed culture medium. The cells were allowed to adhere overnight and replaced with fresh culture medium.

2.2 Intracellular Calcium Measurement

Intracellular calcium levels were measured in KB-60, CHO, CHO PAR-2, N1LF, N1LF PAR-1and N1LF PAR-4 cells as well as neutrophils and platelets. KB-60, CHO, CHO PAR-2, N1LF, N1LF PAR-1, N1LF PAR-4 cells were detached from culture dishes by treatment with non-enzymatic dissociation solution (Sigma). All cell types were prepared for $[Ca^{2+}]_i$ measurements as described (Bootman *et al.*, 1992). Cells were washed and resuspended at 6×10^6 cells/mL in an extracellular medium (EM) the composition of which depended on the cell type. In all subsequent steps the cells were protected from light. Cells were loaded with the calcium sensitive dye Fura-2 AM at 1 μM (Grynkiewicz et al., 1985) by occasional shaking for 30 min at room temperature. After centrifugation at 200 × *g* for 5 min, cells were resuspended in EM incubated for 30 min at room temperature with occasional shaking to allow hydrolysis of the intracellular Fura-2 AM, and then centrifuged (200 x *g* for 5 min). KB-60, CHO, CHO PAR-2, N1LF, N1LF PAR-1, N1LF PAR-4 cells and neutrophils were resuspended in EM without BSA at 2×10^6 cells/mL for fluorescence measurements. Platelets were resuspended in EM for platelets containing 1mM $CaCl_2$. $[Ca^{2+}]_i$ was determined using a Perkin Elmer LS-50 fluorimeter. Fura-2 fluorescence was measured at excitation and emission wavelengths of 340/380 and 510 nm, respectively. Loaded cells were maintained at 37°C in stirred plastic cuvettes throughout the experiment. After a stable baseline was established, the agonist was added to cells and the ratio of fluorescence at the two excitation wavelengths was measured, which is proportional to $[Ca^{2+}]_i$.

EM for KB-60, CHO, CHO PAR-2, N1LF, N1LF PAR-1, N1LF PAR-4, and neutrophils: 121 mM NaCl, 5.4 mM KCl, 0.8 mM $MgCl_2$, 1.8 mM $CaCl_2$, 6 mM $NaHCO_3$, 5.5 mM glucose, 25 mM Hepes, 0.1% (w/v) BSA, pH 7.3.

EM for platelets: 12 mM $NaHCO_3$, 138 mM NaCl, 5.5 mM glucose, 2.9 mM KCl, 10 mM HEPES, 1mM $MgCl_2$, pH 7.4.

2.2.1 Data Analysis

The dependence of the increase in $[Ca^{2+}]_i$ on the concentration of the agonist added to the cells was fitted to a one site binding hyperbola by non-linear regression to estimate the maximal response and the concentration of the agonist causing a half maximal response (EC_{50}).

2.3 Purification of Neutrophils from Human Blood

Neutrophils were isolated from whole blood as described previously (Jepsen and Skottun. 1982). Percoll (Pharmacia) was diluted 9:1 with 1.5 M NaCl (Percoll stock). 78% (v/v) Percoll was prepared by diluting 50.7 mL of the Percoll stock with 14.3 mL normal saline (0.15 M NaCl), and 54% (v/v) Percoll by diluting 24.3 mL Percoll stock with 20.7 mL normal saline. Gradients were constructed by layering 2 mL of 54% (v/v) Percoll onto 3 mL 78% (v/v) Percoll in 20 mL tubes. Venous blood (30 mL) was collected from healthy volunteers into lithium heparin tubes, diluted 1:1 with normal saline and 5 mL was layered onto the Percoll gradient. The tubes were centrifuged at 200 × *g* for 35 min at room temperature. Polymorphonuclear cells were harvested from the interface of the two Percoll gradients. Cells were diluted 5-10 fold in EM and centrifuged at 300 × *g* for 15 min. If

necessary, the cell pellet was resuspended in ice cold erythrocyte lysis buffer (8.275 g NH_4Cl, 1 g $NaHC0_3$, 0.0372 g EDTA in 1 L ddH_20) allowing contaminating erythrocytes to lyse on ice for 5 min, and then centrifuged at 4°C. The pellet was resuspended in EM. To assess the purity of the neutrophil preparation, 100 µL of cells were added to 100 mL of PBS and placed in the chamber of a cytospin apparatus and centrifuged at 50 × g for 5 min onto a glass slide. Slides were dehydrated in methanol and stained with Giemsa for morphological identification.

2.4 Purification of Platelets from Human Blood for Intracellular Calcium Measurements

Platelets were isolated from freshly drawn human blood. Venous blood was anti-coagulated by adding 6 volumes of blood to 1 volume acid-citrate-dextrose (85 mM sodium citrate, 111 mM dextrose and 71 mM citric acid supplemented with PGI_2 (50 ng/mL) and apyrase (0.67 U/mL). Whole blood was centrifuged at 200 × *g* for 20 min and the supernatant of platelet rich plasma was obtained. The plasma was centrifuged at 730 × *g* for 10 min to sediment the platelets. The platelet pellets were resuspended in CGS (13 mM trisodium citrate, 120 mM NaCl and 30 mM dextrose pH 7.0), containing PGI_2 (50 ng/mL) and washed twice. Platelets were resuspended in EM (12 mM $NaHCO_3$, 138 mM NaCl, 5.5 mM glucose, 2.9 mM KCl, 10 mM Hepes, 1 mM $MgCl_2$, pH 7.4).

2.5 Platelet Aggregation Studies

2.5.1 Purification of Platelets from Human Blood for Platelet Aggregation Studies

Platelets were collected from healthy volunteers who had not taken anti-platelet medication for two weeks. Blood was collected in acid-citrate-dextrose, 6:1 v/v, containing 90 mM sodium citrate, 7 mM citric acid, pH 4.6, 140 mM dextrose, supplemented with 70 mM theophylline. Platelet rich plasma (PRP) was obtained by centrifugation of whole blood at 180 x *g* for 15 minutes. PRP was centrifuged at 730 x *g* for 15 minutes and the platelets washed twice with buffer containing 4.3 mM Na_2HPO_4, 4.3 mM K_2HPO_4, 24.3 mM NaH_2PO_4, pH 6.5, 113 mM NaCl, 5.5 mM glucose, 5 mg/mL bovine serum albumin (BSA) and 10 mM theophylline. The final platelet preparation was resuspended in a modified Tyrode's buffer consisting of 12 mM $NaHCO_3$, 0.32 mM NaH_2PO_4, 10 mM HEPES, pH 7.5, 137 mM NaCl, 2.7 mM KCl, 0.5 mM $MgCl_2$ and 5.5 mM glucose.

2.5.2 Platelet Aggregation Studies

Platelet aggregation was performed using a four-channel automated platelet analyzer (Kyoto Daiichi, Japan) set to 950 rpm at 37°C. Each reaction mixture (400 µL) contained washed platelets (3 x 10^8/mL) and the indicated concentrations of HRgpA, RgpB or thrombin, in the presence of 1 mM Ca^{2+} and in the absence of fibrinogen. The rate and extent of platelet aggregation were monitored by the percentage of light transmission and presented as aggregation tracings.

2.6 RNA Isolation

RNA was isolated using the TRI reagent (Sigma) according to the manufacturer's instructions. ddH_20 was treated with 0.1% (v/v) DEPC in glassware at 37°C overnight and autoclaved the next day. Pipette tips and microcentrifuge tubes were covered with ddH_20 containing 0.1% (v/v) DEPC and incubated at 37°C overnight and then autoclaved. All solutions used were poured into sterile 50 mL Falcon tubes.

Adherent cells (grown to confluence) were washed twice with PBS. TRI reagent was then added to the cells (approximately 1 mL of TRI reagent per 10 cm^2 surface area) and cells were homogenised by pipetting. The homogenate was aliquoted in microcentrifuge tubes in 1 mL aliquots. Chloroform (200 µL) was added to every 1 mL of TRI reagent and the mixture was vortexed for 20 sec and then kept at room temperature for 10 min. It was then centrifuged at 13,000 rpm at 4°C for 15 min. The aqueous layer was then transferred to a microcentrifuge tube and 500 µL of isopropanol was added. The microcentrifuge tube was inverted, incubated at room temperature for 10 min and then centrifuged at 13,000 rpm at 4°C for 10 min. The supernatant was removed and the RNA pellet was resuspended in 1 mL of 75% (v/v) ice cold ethanol, vortexed for 20 sec and centrifuged at 13,000 rpm at 4°C for 10 min. The pellet was allowed to air dry following which it was resuspended in DEPC-treated ddH_20. The concentration of the RNA was determined by measuring the absorbance at 260 nm, on the basis that RNA at a concentration of 40 mg/mL has an OD of 1.0 at 260 nm (Sambrook *et al.*, 1989). The purity of the isolated RNA was evaluated by determining the $A_{260/280}$ ratio. The RNA was aliquoted and stored at –80°C.

2.7 Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

2.7.1 RT- PCR for the determination of expression of protease-activated receptors in KB-60 cells

RT-PCR was carried out using the Ready to Go U Prime Synthesis beads (Pharmacia) according to the manufacturer's instructions. For the RT step, RNA (5 μg), dT primer (40 pmol) and DEPC-treated ddH_20 to a volume of 33 μL were added to a microcentrifuge tube containing the Ready to Go U Prime Synthesis beads and incubated at 37°C for 1 hr. The reaction mix was then heated at 65°C for 5 min. For the PCR step, the appropriate forward and reverse primers (20 pmol for PAR-2,-3,-4 and 100 pmol for PAR-1 primers), 2.5 units of Taq polymerase (MBI Fermentas), milli-Q H_20 to a final volume of 100 μL were added to the entire reaction mixture from the RT step. A control reaction, containing RNA in a RT reaction mix that had not undergone reverse transcription, was used when the primers did not span an intron.

PCR was carried out using a Thermal Sequencer (Corbett Research, PC-9606C). For amplification of PAR-2, -3, and -4 products, PCR was performed for 36 cycles. The first cycle included a denaturation step for 5 min at 95°C. Cycles 2-36 had a denaturation step of 1 min at 95°C, 1 min annealing at 55°C and 3 min elongation at 72°C. For PAR-1 amplification, PCR was performed for 34 cycles. The first cycle included a denaturation step for 2min at 95°C. Cycles 2-34 had a denaturation step of 1 min at 95°C, 1 min annealing at 55°C and 1min elongation at 72°C.

2.7.2 Oligonucleotides used for RT-PCR

Oligonucleotide	Sequence 5'-3'	annealing bp	Reference
PAR-1 S	TGTGAACTGATCATGTTTATG	2421	Vu *et al.*, 1991
PAR-1 AS	TTCGTAAGATAAGAGATATGT	3129	Vu *et al.*, 1991
PAR-2 S	GCAGCCTCTCTCTCCTGCAGTGG	48	designed
PAR-2 AS	CTTGCATCTGCTTTACAGTGCG	1114	Storck *et al.*, 1996
PAR-3 S	ATAACGTTTAAGAGACGGGACT	111	Schmidt *et al.*,1998
PAR-3 AS	TAGCAGTAGATGATAAGCACA	969	Schmidt *et al.*,1998
PAR-4 S	GACGAGAGCGGGAGCACC	195	Xu *et al.*,1998
PAR-4 AS	CCCGTAGCACAGCAGCATGG	970	Xu *et al.*,1998
β-actin S	ACCTTCAACACCCCAGCCATGTACG	1076	Kakinuma *et al.*,1998
β-actin AS	CTGATCCACATCTGCTGGAAGGTGG	376	Kakinuma *et al.*,1998
GAPDH S	ACCACCATGGAGAAGGCTGG	381	Tso *et al.*, 1985
GAPDH AS	CGTAGGACCCGATGTGACTC	890	Tso *et al.*, 1985
dT	TTTTTTTTTTTTTTT		

S: sense, AS: antisense

2.8 ELISA for the Secretion of IL-6, TNFα and IFN-γ by KB-60 cells

KB-60 cells were grown in six-well plates and treated in duplicate with proteases or peptide receptor agonists in medium lacking FCS. Following treatment, the cells were washed with medium and grown in medium containing 5% (v/v) FCS for 24 hours. The supernatant from the KB-60 cells was then removed, centrifuged, aliquoted and stored at –80°C.

The ELISA was carried out using an ELISA kit (Endogen, CSL Biosciences) at room temperature, according to the manufacturer's instructions. A 96-well plate was coated

(100 μL/well) overnight with the coating antibody diluted in PBS at a concentration of 2.5 μg/mL, 3.5 μg/mL and 2.5 μg/mL for the IL-6, TNFα and IFNγ antibodies, respectively. The coating antibody was removed and the plate was blocked with assay buffer 200 μL/well (4% (w/v) BSA in PBS) for 30 min, followed by washing three times with washing buffer (50mM Tris, 0.2% (v/v) Tween-20, pH 8.0). For the IL-6 ELISA, 50 μL of supernatant was added to each well, followed by 50 μL of IL-6 biotinylated anti-IL-6 (detecting) antibody in assay buffer at a concentration of 0.3 μg/mL for 2 hours. A standard curve was obtained by adding 50 μL of purified IL-6 protein to each well, diluted in medium, in the range of 15-500 pg/mL. For the TNFα and IFNγ ELISA, 50 μL of supernatant was added to each well. One hour later, 50 μL of biotinylated anti-TNFα or biotinylated anti-INFγ (detecting) antibody at a concentration of 0.6 μg/mL was added to each well and left for one hour. Purified TNFα or IFNγ proteins, diluted in medium, in the range of 15-500 pg/mL (50 μL/well) were used to produce a standard curve. The plate was washed three times with wash buffer and 100 μL of HRP-conjugated streptavidin (diluted in assay buffer 1:14,000 for IL-6 and IFN-γ and 1:30,000 for TNFα) was placed in each well for 1 hr. The plate was washed twice, and TMB substrate solution (Sigma) (100 μL) was added to each well and incubated for 30 minutes. The reaction was stoped by the addition of 100 μL of 0.18 M H_2SO_4. The absorbance was read at 490 nm using a microtitre plate reader (Thermomax, Molecular Devices). The concentration of the proteins in the supernatant of the unknown solutions was determined from the standard curves.

2.9 Cell-ELISA for integrin β_1 expression

KB-60 cells were seeded in a 96-well flat-bottomed tissue culture plate (4×10^4 or 10×10^4 cells/well) in 0.2 mL of medium containing FCS, and 24 hrs later were treated with trypsin or RgpB in medium lacking FCS for various times. The cells were then washed with medium. The cells were washed twice with PBS containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$ (PBS^{++}) and fixed for 15 min using 1% (v/v) paraformaldehyde in PBS^{++}. Following two washes with PBS^{++}, the cells were incubated with PBS^{++} containing 0.2% (w/v) BSA for 20 min. The cells were then incubated with mouse anti-human integrin β1 monoclonal antibody (2 μg/mL: Chemicon; 200 μL/well) at room temperature for 2 hrs and washed twice with PBS^{++}. Horseradish peroxidase-labelled anti-mouse immunoglobulin (1:4,000, Silenus) was added (200 μL/well) to the cells for 40 min. The cells were washed three times with PBS^{++}. Substrate (tetramethylbenzidine dihydrochloride, Sigma) was dissolved at 0.1 mg/mL in substrate buffer (0.1 M Na_2HPO_4, 0.05 M citric acid, pH 5) and 2 μL of 30% (v/v) H_20_2 was added per 10 mL of substrate solution prior to use. Substrate solution (200 μL) was added to each well. When colour was developed the reaction was stopped with 50 μL 2 M H_2SO_4 and read at 450 nm using a spectrophotometric microtitre plate reader (Thermomax, Molecular Probes).

2.10 Immunocytochemistry

KB-60 cells, grown to near confluence in 8-chamber slides, were treated with various concentrations of RgpB in medium lacking FCS and following treatment were washed with medium. The cells were washed twice with PBS containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$ (PBS^{++}), fixed for 15 min in 1% (v/v) paraformaldehyde in PBS^{++} and washed twice with

PBS^{++}. Non-specific binding sites were blocked with PBS^{++} containing 0.2% (w/v) BSA for 20 min at room temperature. The cells were then incubated with mouse anti-human integrin β1 monoclonal antibody (2 μg/mL) at room temperature for 2 hrs. Following two washes with PBS^{++}, rhodamine-labelled anti-mouse immunoglobulin (1:300, Dako, Australia) was added to the cells for 45 min at room temperature. The cells were washed twice with PBS^{++} and stained with the nuclear stain DAPI (0.1 μg/mL) in PBS^{++} for 5 min and then washed with PBS. The slide was coverslipped with mounting medium (DAKO) and stored at 4 °C. Cells were visualised and photographed using an Olympus Vanox AHBT3 photomicroscope.

2.11 FACS Analysis

Samples (100 μL) of human blood anticoagulated with heparin were placed in tubes in duplicate and incubated for 15 min at 37°C with different reagents: RAP (300 μM), fMLP (100 nM), buffer alone, RgpB (50 nM) activated in cysteine buffer, cysteine buffer alone. To each sample CD11b-PE conjugated antibody (20 μL) was added. Samples were incubated on ice for 15 min then an aliquot of 10 μL was removed and added to 490 μL cold PBS containing the fluorescent nuclear dye LDS-751 (20 μg/mL). These samples were placed on ice for 10 min before flow cytometric analysis using a FACS IV (Becton Dickinson, Mountain View, CA).

Spectral compensation was made for PE emissions entering the FL3-H (LDS-751) channel. Lymphocytes, monocytes and granulocytes were gated on the fluorescence intensity of LDS-751 (FL3-H) and side light scatter. A second gate was set for granulocytes, distinguished from lymphocytes and monocytes on the basis of FL3-H and

side light scatter characteristics. Median fluorescence intensities of PE emissions (FL2-H) were recorded.

2.12 Myeloperoxidase Enzyme Assay

Myeloperoxidase release was measured by an adaptation of the method described by Lundberg and Arfors (1983). Isolated human neutrophils were resuspended in Earles balanced salt solution buffered with 10 mM MOPS buffer and centrifuged at 200 × *g* for 10 min. The neutrophils were washed again in the above-mentioned buffer and resuspended at a concentration of 20 x 10^6 cells/mL in the same buffer containing 5 μg/mL of cytochalasin B and incubated at 37°C for 15 min. Samples (250 μL) of neutrophils were placed in tubes in duplicate and incubated in the presence of 10 mM cysteine for 10 min at 37°C with different reagents: PMA (60 nM), trypsin (50 nM), RAP (300 μM), RgpB (50 nM) and cysteine alone. The samples were centrifuged (200 × *g* for 10 min) and supernatant from each treatment (25 μL) was added to 150 μL of 0.01 M phosphate buffer, pH 6.5. Immediately prior to reading the assay 0.33 μL 0.3% (v/v) H_2O_2 and 25 μL 1.784 mg/mL O-dianisidine-DiHCl was added. The change in absorbance was read at 450 nm using a spectrophotometric microtitre plate reader (Thermomax, Molecular Probes).

2.13 Enzyme Assays and Measurement of Kinetic Constants

The fluorescence quenched peptides Abz-Leu-Asp-Pro-Arg-Ser-Phe-Leu-Leu-Lys(Dnp)-Asp-OH (PAR-1) and Abz-Ser-Lys-Gly-Arg-Ser-Leu-Ile-Gly-Lys(Dnp)-Asp-OH (PAR-2) were synthesised using standard solid phase 9-fluorenylmethyloxycarbonyl (Fmoc) chemistry with an automated peptide synthesizer (Applied Biosystems, model 432A) by Dr P. Thompson (Dept. Medicine, Monash University). They were purified using reverse-phase high performance liquid chromatography and their structure was confirmed by mass spectroscopy by Dr Thompson.

Experiments were carried out in 0.1 M Hepes, 0.1 M NaCl, 10 mM $CaCl_2$, 0.2% (w/v) polyethylene glycol (M_r 6000), pH 7.4. The substrate solution (0.5 mL) was allowed to equilibrate to 37°C for 10 min, enzyme solution was added, and enzyme activity monitored by continuously measuring the fluorescence (λ_{ex}= 325 nm; λ_{em}= 414 nm [10 nm slits]) in a Perkin Elmer LS-50B spectrofluorometer (Chagas *et al.*, 1991).

Lyophilised fluorescence quenched PAR-2 peptide was resuspended in dimethylformamide, and the concentration of the stock solution (6.4 mM) determined spectrophotometrically, assuming an absorption coefficient of 10^4 M^{-1} cm^{-1} at 360 nm (Chagas *et al.*, 1991). Fluorescence of peptide products after exhaustive cleavage by trypsin was found to be proportional to concentration. Accordingly, an increase in fluorescence could be equated to the increase in concentration of the cleaved substrate, allowing determination of the kinetic parameters, K_m and V_{max} from an analysis of initial velocities obtained at different substrate concentrations. Values for k_{cat} were determined from Vmax values by taking the concentrations of active enzyme used into account ($k_{cat}=V_{max}/[E]_o$).

2.14 SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was carried out using the Mini-Protean II dual slab gel apparatus (Bio-Rad). The resolving gel contained 0.375 M Tris-HCl, pH 8.8, 0.1% (w/v) SDS, 10% (w/v) NN'-bismethylene acrylamide (30:80), 0.05% (w/v) ammonium persulphate, 0.1% (v/v) TEMED and milli-Q water to a final volume of 5 mL for each gel. After being poured into the casting sandwich, the gel was overlayed with water and allowed to set for 30 min. The stacking gel consisted of 0.125 M Tris-HCl, pH 6.8, 0.1% (w/v) SDS, 4% (w/v) NN'-bismethylene acrylamide (30:80), 0.05% (w/v) ammonium persulphate, 0.1% (v/v) TEMED and milli-Q water to a final volume of 2.5 mL for each gel. The water was poured off the resolving gel and then the stacking gel was poured on. A gel comb was inserted in the stacking gel and left to set for 30 min. The casting sandwich containing the gel was placed in the tank of the running apparatus filled with SDS-PAGE running buffer (25 mM Tris, 192 mM glycine, 0.1% (w/v) SDS). The samples were denatured in 10x sample buffer [0.1 M Tris HCl pH 6.8, 10% (w/v) SDS, 0.04% (w/v) bromophenol blue, 20% (v/v) glycerol, 5.5% (v/v) β–mercaptoethanol] by heating at 95°C for 5 min. The gel was electrophoresed at constant voltage of 200V.

2.14.1 Coomassie Brilliant Blue Staining

Gels were stained for 2 hrs in a staining solution containing 40% (v/v) methanol, 10% (v/v) acetic acid, 0.25% (v/v) Coomassie brilliant blue dye. The gel was then destained with destaining solution consisting of 40% (v/v) methanol and 10% (v/v) acetic acid.

2.14.2 Silver Staining

The Silver Stain Plus kit (BioRad) was used. The gel was placed in 200 mL of fixative enhancer solution (50% (v/v) methanol, 10% (v/v) acetic acid, 10% (v/v) fixative enhancer concentrate, 30% (v/v) dH_20) for 20 min. The fixative enhancer solution was removed and the gel was then washed with 300 mL of dH_20 for 10 min. This wash step was repeated twice. The silver stain solution was prepared within 2 min of use by adding 5 mL of silver complex solution, 5 mL of reduction moderator solution and 5 mL of image development reagent to 35 mL of dH_20, and finally adding 50 mL of development accelerator solution that was continuously being stirred. The gel was placed in the silver stain solution with gentle agitation. When sufficient staining was achieved, the staining solution was removed and the gel was placed in 5% (v/v) acetic acid to stop the staining reaction.

2.15 Western Blotting

Following SDS-PAGE, the gel was removed from the electrophoresis apparatus and the stacking gel was discarded. The gel was then placed in a Bio-Rad Mini Blot transfer sandwich, which was assembled by using a sponge, 3 pieces of blotting paper (3MM), the gel, a piece of nitrocellulose membrane, 3 pieces of blotting paper and another sponge. Air bubbles between each layer were removed. The sponges, the paper and the membrane were pre-soaked in transfer buffer (25 mM Tris, 192 mM glycine, 20% (v/v) methanol). The sandwich was then placed in an electrophoresis tank, so the gel was closer to the negative electrode; an ice pack and a magnetic stirrer were added and the tank was filled with cold transfer buffer. The transfer of proteins from the gel to the nitrocellulose was carried out at 150 mA constant current for 2 hrs while the buffer was constantly stirred. Following

disassembly of the apparatus the membrane was blocked with Blotto/PBS (5% (w/v) skim milk powder in PBS) at RT for 1 hr. The gel was then placed in a plastic bag containing anti- RgpB antibody diluted 1:1000 in Blotto/PBS. Any bubbles were removed from the bag, which was then heat sealed and incubated overnight at 4°C on a shaker. The membrane was washed 3 times with Blotto/PBS and then incubated with the alkaline phosphatase conjugated anti-mouse immunoglobulin diluted (1:1000) in Blotto/PBS, for 2 hrs at RT. Antibody was detected using the BCIP/NBT (Sigma) substrate according to the manufacturer's instructions. A tablet was dissolved in 10 mL of dH_20, poured over the nitrocellulose membrane and the nitrocellulose was removed when sufficient colour was developed.

2.16 Purification of RgpB from *Porphyromonas gingivalis*

2.16.1 Strain

The anaerobic bacterial strain *Porphyromonas gingivalis* H66 was used for the purification of gingipain-R. *P. gingivalis* H66 produces a soluble form of gingipain-R (RgpB) that is secreted in the culture medium.

2.16.2 Media

Liquid media were prepared by dissolving the appropriate substances in distilled water. The media contained 3% (w/v) trypticase soy broth, 0.5% (w/v) yeast extract and 0.05% (w/v) L-cysteine. The media was aliquoted in round bottom flasks and heated to boiling point. The flasks were then immediately placed on ice, and gas mixture (85%N_2,

10% CO_2, 5% H_2) was bubbled in to the media. Plastic corks were placed on the flasks and set in place using wire. These were then autoclaved for 20 min at 120°C and 100 kPa. Media was stored at room temperature. Prior to use, the media was supplemented with 0.1% (w/v) Vitamin-K (Vitamin-K stock: 1 mg/mL in ethanol) and 0.0005% (w/v) hemin (hemin stock: 100 mg/mL in 1 M NaOH, filter sterilised) which was injected into the media through the plastic cork with the use of a syringe, following which the cork was flamed.

2.16.3 Maintenance and Propagation of Bacterial Strain

Stocks of *P. gingivalis* that had been frozen in whole human blood (provided by Dr Jan Potempa, UGA, USA) were stored at −80°C. An aliquot (1 mL) of frozen *P. gingivalis* stock was thawed and used to inoculate 25 mL of media. The 1 mL aliquot was injected into the media through the plastic cork using a syringe and the cork was then flamed. Following 4 days of growth, the 25 mL culture was immediately used for the inoculation of a 1 L culture to be used for purification of gingipain-R. A 25 mL culture of *P. gingivalis* was stored at 4°C for up to 1 month and was used to inoculate fresh cultures.

2.16.4 Purification of RgpB

RgpB was purified from the culture medium of *P. gingivalis* as previously described (Potempa et al. 1998). The culture fluid (2 L) was centrifuged at 6000 × *g* for 30 min at 4°C. The supernatant was obtained and placed in a dry ice and ethanol bath, ensuring that the temperature remained below 0°C. Chilled acetone (3 L) was added dropwise to the supernatant. This mixture was then centrifuged at 6000 × *g* for 30 min at −15°C). The precipitate was redissolved in 200 mL of buffer A (20 mM Bis Tris-HCl, 150 mM NaCl,

5 mM $CaCl_2$, 0.02% (w/v) NaN_3, pH 6.8) and dialysed against buffer A (2 changes) overnight. The dialysed fraction was centrifuged at 27000 × *g* for 30 min at 4 °C and then it was concentrated using an Amicon PM-10 membrane.

This concentrated fraction was applied to a Sephadex G-150 column (90 x 5 cm, 1765 mL) previously equilibrated with buffer A, and the fractionation was carried out at 0.8 mL/min. Fractions (12 mL) were assayed for activity against Bz-L-Arg-pNA and Z-L-Lys-pNA. Two peaks with activity against Bz-L-Arg-pNA were found with the lowest molecular weight peak having the majority of activity and no activity against Z-L-Lys-pNA. The lowest molecular weight peak was pooled and dialysed overnight against two changes of buffer B (20 mM Bis Tris-HCl, 1 mM $CaCl_2$, 0.02% (w/v) NaN_3, pH 6.4).

Using a Bio Rad HR chromatography system this lowest molecular weight peak was applied to a mono Q column (1 mL), equilibrated with buffer B, and washed with 5 column volumes of buffer B at 1mL/min. The protein was then eluted using a linear gradient from 0-0.5M NaCl over 60 min. The majority of the activity was eluted at 0.1 M NaCl. The active fractions were pooled and dialysed overnight against two changes of Buffer C (50 mM Tris-HCl, 1 mM $CaCl_2$, 0.02% (w/v) NaN_3, pH 7.4).

The active fraction from the mono Q column was then applied to an arginine-Sepharose column (1.6 x 12.5 cm, 25 mL), previously equilibrated with Buffer C at a flow rate of 0.4 mL/ min. The column was then washed with Buffer B until activity against BAPNA was below .10 mOD/min and the remaining proteins were eluted with 500 mM NaCl in Buffer C until the A_{280} reading was zero . Three peaks of activity were obtained at this stage: non absorbed (A) and retarded (B and C) and eluted with NaCl (D), with peak B having the majority of the activity. The column was then washed with 200 mM arginine in Buffer C at the same flow rate and another peak of activity was eluted.

The activity peak B was dialysed overnight against two changes of buffer D (50 mM Na_2COOCH_3, 5 mM $CaCl_2$, 0.02% (w/v) NaN_3, pH 4.5).

Using a Bio Rad HR system, peak B from the arginine-sepharose column was applied to a mono S column, equilibrated with buffer D, and washed with 5 column volumes of Buffer D at 1 mL/min. The protein was then eluted using isocratic and linear increases in [NaCl] over the range of 0-0.25 M. The majority of the activity was eluted at 0.15 M NaCl. The fractions from the Mono S column that displayed activity against the Bz-L-Arg-pNA substrate were pooled then analysed by SDS-PAGE and visualised using Coomassie Blue and Silver Staining. The fractions contained a single band of the expected molecular weight (48.3 kDa) for RgpB. Following a Western Blot probed with an anti-RgpB antibody this band was verified as being RgpB. The enzyme was aliquoted and stored at –80°C.

2.16.5 Activation of gingipains-R

Gingipains-R were incubated in activation buffer (0.2M Tris-HCl, 1mM $CaCl_2$, 10 mM cysteine, pH 7.6 filter sterilised) at 37°C for 10 min. When the enzymes were used for treatment of cells, Polymyxin B sulfate (100 µg/mL) was also added to inhibit any lipopolysaccharide activity that may be present.

2.16.6 Activity Assay of gingipains-R

The amidolytic activity of the gingipains was determined using Benzoyl-Arg-*para*nitroanilide (Bz-L-Arg-pNA; 0.5 mM) as a substrate. Samples were incubated in activation buffer (0.1 M Tris-HCl, 0.1 M NaCl, 5 mM $CaCl_2$, 10 mM cysteine

[added immediately before use from a 1 M cysteine stock solution], pH 7.6) at 37°C for 10 min. The assay was done in a 96 well plate. Following incubation of the samples with the buffer at 37°C, the substrate was added and the plate was immediately read at 450 nm using a microtitre plate reader (Thermomax, Molecular Probes).

2.16.7 Active site titration of gingipains-R

The concentration of active RgpB and HRgpA was calculated by active site titration of the enzymes with the inhibitor FPRck. The enzyme was diluted to 2 μM (based on protein estimation) in activation buffer (0.2 M HEPES, 2 mM $CaCl_2$, 10 mM cysteine, pH 7.6) and incubated at 37°C for 5 min. Immediately before use the inhibitor FPRck was diluted (eg. 0, 0.25, 0.5, 1.0, 1.25 μM FPRck) in 0.1 M HEPES, 2mM $CaCl_2$, pH 7.6. Aliquots (95 μL) of the different inhibitor concentrations were added to 5 μL of activated enzyme and incubated at room temperature for 15 min. Aliquots (5 μL) of the enzyme-inhibitor mix were taken and assayed as described above. The activity of the enzyme was plotted against the inhibitor concentration and assuming that the enzyme to inhibitor ratio is 1:1, the concentration of the enzyme was calculated.

CHAPTER 3

RgpB from *Porphyromonas gingivalis* Cleaves and Activates PAR-2 on Human Neutrophils

3.0 Introduction

Periodontal disease is an inflammatory disease characterised by neutrophil infiltration and accumulation in the gingival tissue and periodontal pocket (Attstrom *et al.*, 1970; Schenkein, 1999; Page, 1998). Proteases produced by *P. gingivalis* called gingipains-R (Rgps), have been shown to have a profound disruptive effect on host systems, particularly those controlling inflammatory events. Rgps have been demonstrated to enhance vascular permeability (Imamura *et al.*, 1994) and activate the complement system (Wingrove *et al.*, 1992; DiScipio *et al.*, 1996) thus contributing to the inflammation seen in periodontal disease. Rgps cleave complement factor C5 to yield C5a-like fragments with neutrophil chemoattractant activity (Wingrove *et al.*, 1992; DiScipio *et al.*, 1996) and in addition cleave IL-8 to produce an N-terminal truncated product, which had three-fold higher neutrophil activating ability than the original IL-8 molecule (Mikolajczyk-Pawlinska *et al.*, 1998). The local production of these chemotactic anaphylotoxins could contribute to the recruitment of neutrophils into the inflamed tissue.

PAR-2 is expressed on human neutrophils and its stimulation leads to neutrophil activation *in vitro* (expression of the activation molecule CD11b and shape changes; Howells *et al.*, 1997) and PAR-2 agonist peptides induce leukocyte rolling and adhesion *in vivo* (Vergnolle, 1999), which may contribute to a neutrophil-induced inflammatory response. Furthermore, PAR-2 deficient mice exhibited a defect in leukocyte rolling

and therefore a delayed onset of inflammation (Lindner *et al.*, 2000). In addition, PAR-2 expression in endothelial cells has been found to be induced by inflammatory cytokines (Nystedt *et al.*, 1996). In order to evaluate the possible role of PAR-2 in inflammation, however, it is necessary to identify physiological and pathophysiological activators of this receptor, after which it will be possible to determine under which conditions activation of PAR-2 on neutrophils is likely to occur. Thus, the questions addressed in this study were whether RgpB could activate PAR-2 on the surface of human neutrophils and elicit an intracellular signal, and thereby modulate biological responses in neutrophils.

3.1 Results

3.1.1 Purification of RgpB from *P. gingivalis*

The bacterium *Porphyromonas gingivalis* (strain H66) was cultivated under anaerobic conditions and the culture medium was used to purify the enzyme. RgpB as previously described (Potempa *et al.*, 1998). The culture fluid was collected and, following acetone precipitation, was subjected to size exclusion chromatography (Sephadex G-150 column). Fractions were assayed for activity against Bz-L-Arg-pNA and Z-L-Lys-pNA. Two peaks (A & B) with activity against Bz-L-Arg-pNA were found, with the lowest molecular weight peak (B) having the majority of activity and very little activity against Z-L-Lys-pNA (Fig. 3.1). The lowest molecular weight peak was pooled and, following anion-exchange chromatography on mono Q, a peak with activity against Bz-L-Arg-pNA was identified (Fig. 3.2). This peak was pooled and applied to an arginine-Sepharose column in order to remove any high molecular weight gingipain-R (HRgpA), which binds to the arginine-Sepharose with very high affinity and is eluted with arginine (Fig 3.3). The column was washed and the remaining proteins were eluted with 500 mM NaCl. Three active fractions of RgpB were obtained: unbound (A), retarded (B & C) and eluted with NaCl (D), as previously reported (Potempa *et al.*, 1998), due to the differing affinities of the different isoforms of RgpB for arginine residues. As previously reported, the unbound fraction (fraction A) contains a truncated product of RgpB (Potempa *et al.*, 1998). The column was then washed with arginine and another peak of material with enzymatic activity, most likely representing HRgpA, was eluted. The majority of the activity against Bz-L-Arg-pNA was detected during the wash step (retarded fraction B) as previously reported (Potempa *et al.*, 1998) and it has been determined that this fraction contains the most abundant isoform of RgpB. This

fraction was applied to a mono S column. The fractions from the Mono S column that displayed activity against the Bz-L-Arg-pNA substrate (Fig. 3.4) were then analysed by SDS-PAGE and visualised using Coomassie blue (Fig. 3.5A) and silver staining (Fig. 3.5B). The fractions contained a single band of the expected molecular weight (48.3kDa) for RgpB, which was verified as RgpB using a Western Blot probed with an anti-RgpB antibody (Fig. 3.5C).

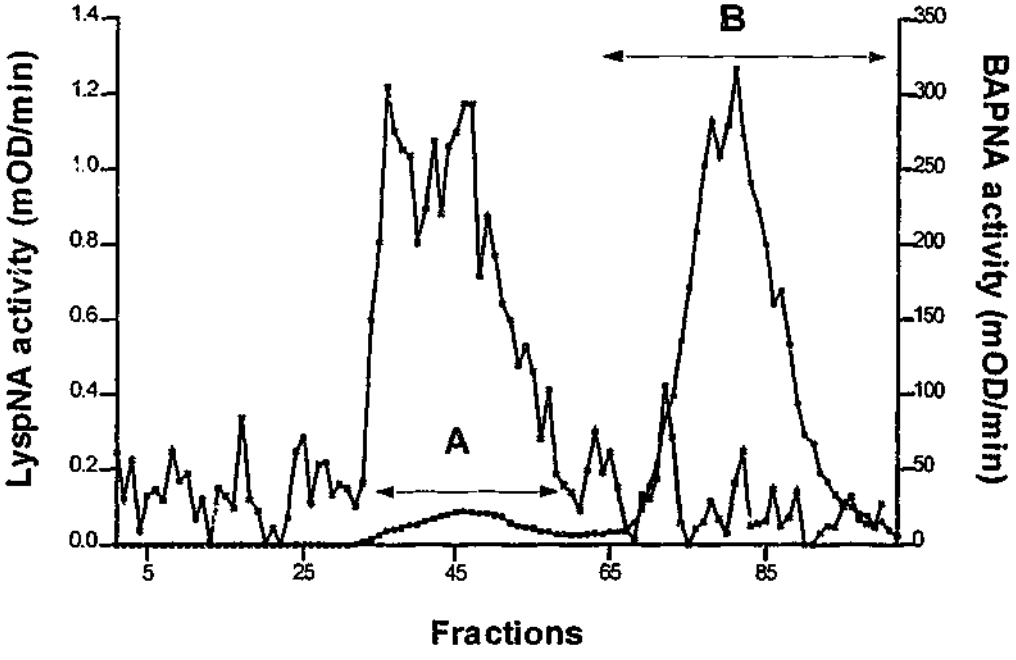


Figure 3.1. **Size exclusion chromatography of acetone precipitate from *Porphyromonas gingivalis*.**

The acetone precipitate obtained from culture fluids was applied to a Sephadex G-150 column. Fractions were assayed for activity against BAPNA (Bz-L-Arg-pNA) (■) and LyspNA (Z-L-Lys-pNA) (×). Activity peaks (A and B) for BAPNA were pooled as indicated by double-headed arrows.

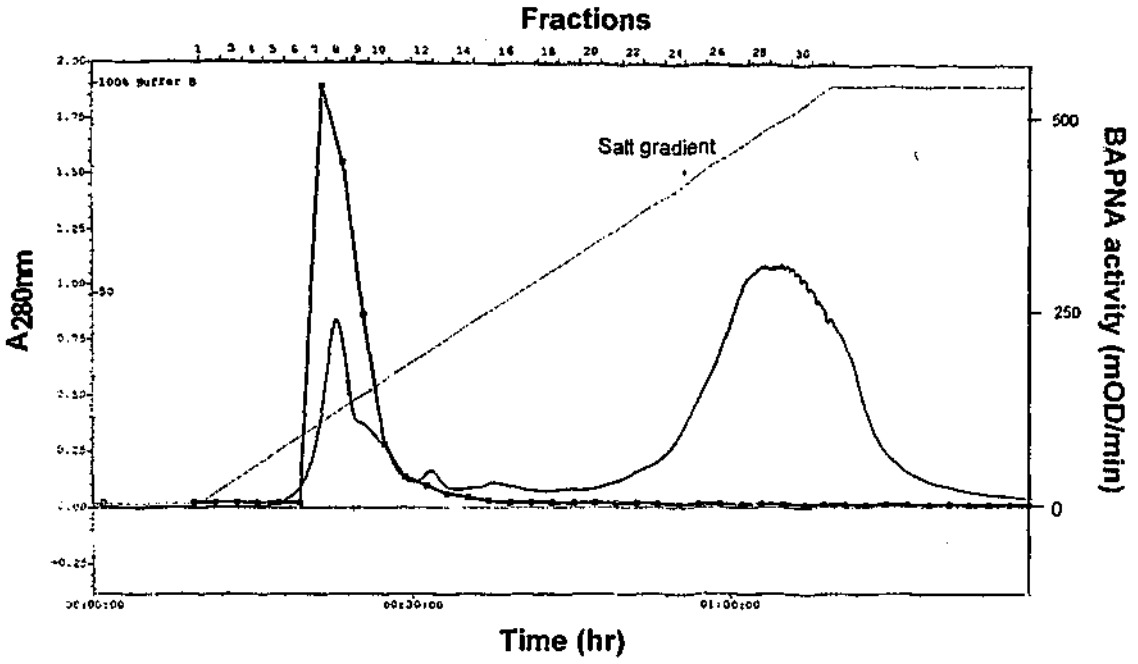


Figure 3.2. **Anion exchange chromatography of R-gingipains.**

The low molecular mass peak (fraction B) from Sephadex G-150 was applied to a Mono Q column. Proteins were eluted using a linear gradient from 0-0.5 M NaCl. Fractions were assayed for activity against BAPNA (Bz-L-Arg-pNA) (▬) and protein content using absorbance at 280nm (—). The majority of the activity was eluted at 100 mM NaCl.

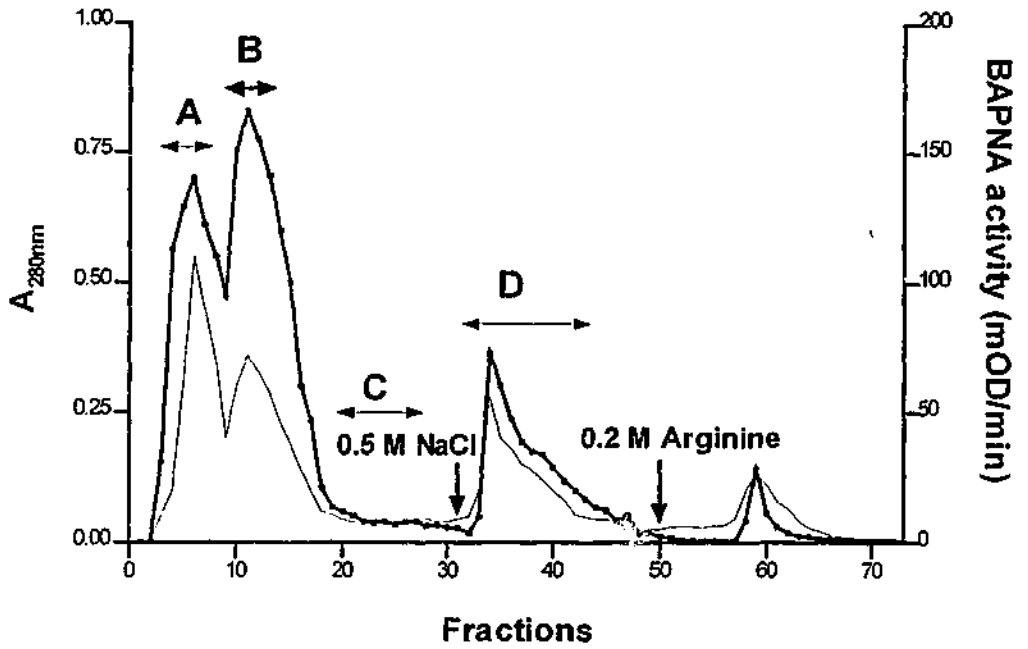


Figure 3.3. **Chromatography of R-gingipains on arginine-Sepharose.**
The active peak from the mono Q column was applied to an arginine-Sepharose column. The column was washed extensively and remaining proteins were eluted with 0.5 M NaCl and 0.2 M arginine. Fractions were assayed for activity against BAPNA (Bz-L-Arg-pNA) (■) and protein content using absorbance at 280 nm (–). Activity peaks (A, B, C and D) were pooled as indicated by double-headed arrows.

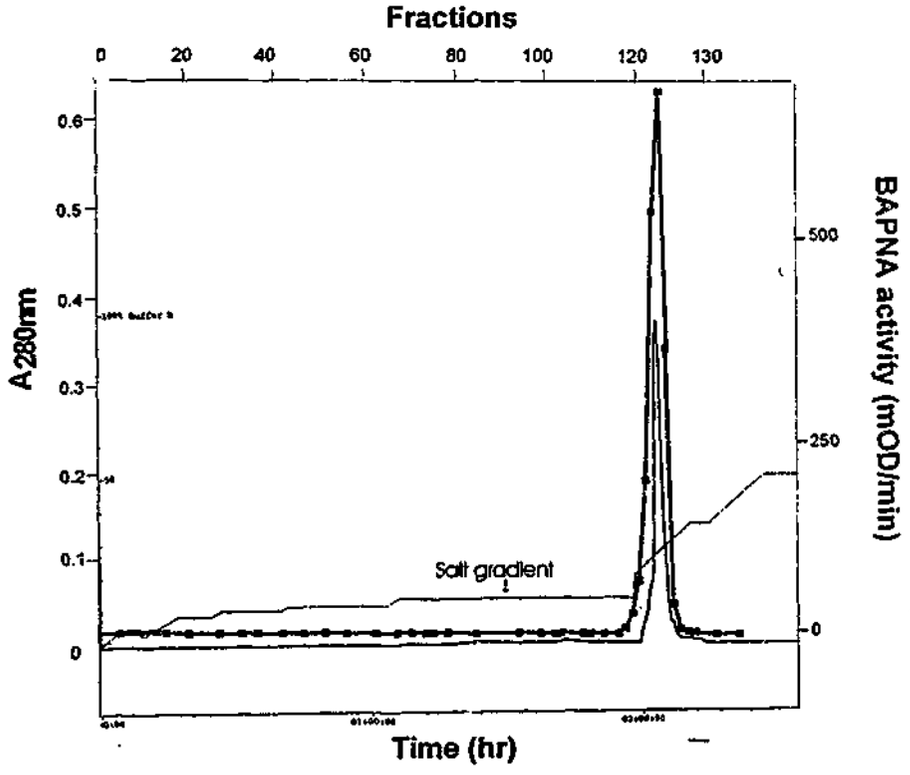


Figure 3.4. **Cation exchange chromatography of RgpB.**

Fraction B from the arginine-Sepharose column was applied to a Mono S column. Proteins were eluted using isocratic and linear increases in [NaCl] over the range 0-0.25 M NaCl. Fractions were assayed for activity against BAPNA (Bz-L-Arg-pNA) (•—) and absorbance at 280nm (–). The majority of the activity was eluted at 0.125 M NaCl.

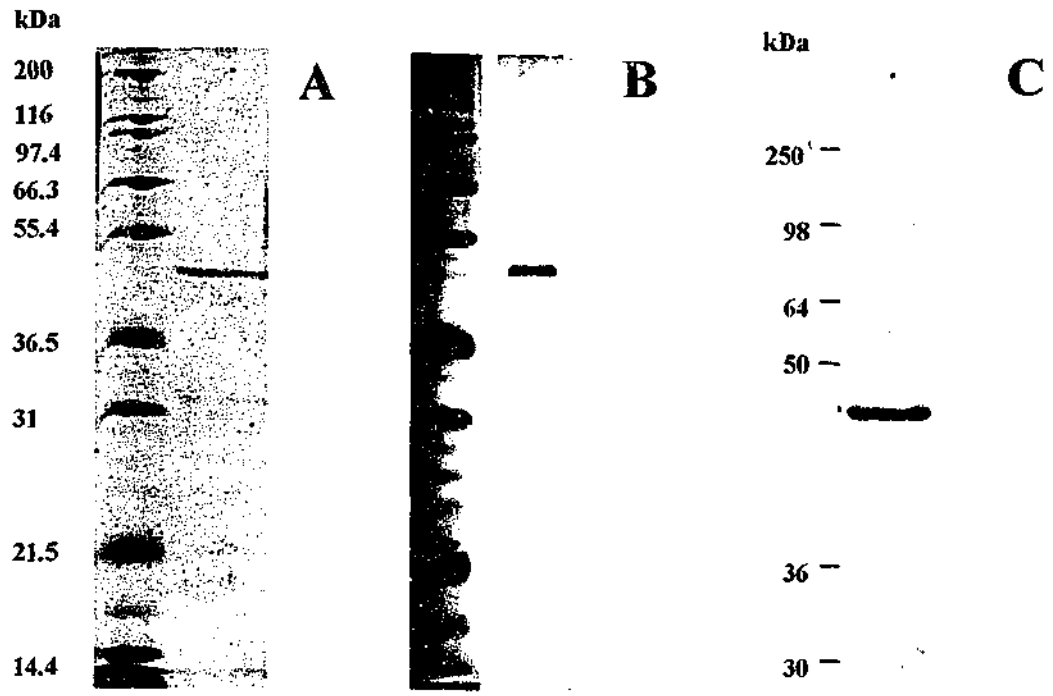


Figure 3.5. **SDS-PAGE and Western Blot analysis of RgpB.**

RgpB from cation chromatography on the mono S column was run on 10% SDS-PAGE and visualised by A) Coomassie stain; B) Silver stain and C) Western blot using RgpB antibodies. The position of molecular weight markers Mark12 Wide Range protein Standards (A &B) and SeeBlue Prestained markers (C) are shown on the left.

3.1.2 RgpB cleaves a synthetic peptide substrate corresponding to the activation sequence of PAR-2

A synthetic peptide, Abz-Ser-Lys-Gly-Arg-Ser-Leu-Ile-Gly-Lys(Dnp)-Asp-OH, which corresponds to the four amino acids on either side of the cleavage site of human PAR-2 (residues 33-41 of human PAR-2), was evaluated for its accuracy as a predictor of receptor cleavage. The Abz group only fluoresces upon release of the Lys(Dnp) quenching group following proteolytic cleavage. Thus, hydrolysis within the PAR-2 activation sequence by a potential receptor activator can initially be assessed in this manner. In order to test this system, cleavage of the peptide was examined with trypsin and thrombin, which are known to be activating and non-activating for PAR-2, respectively. It was found that trypsin cleaved the peptide at concentrations as low as 0.1 nM, while thrombin was incapable of doing so even at significantly higher levels (0.1-100 nM). Trypsin exhibited a high affinity for the peptide as evidenced by a low K_m value (Table 1), and cleaved the peptide efficiently, with a high k_{cat}/K_m value. This cleavage by trypsin, but not thrombin, supports the hypothesis that this peptide may be considered an effective model for determining the potential of a given protease to activate PAR-2. RgpB was examined for its ability to hydrolyse the PAR-2 peptide. RgpB at concentrations as low as 0.1 nM cleaved the test peptide with very similar kinetics to trypsin (Table 3.1), indicating that this bacterial protease has the potential to cleave within the receptor itself.

Table 3.1. **Kinetic parameters of hydrolysis of a synthetic peptide corresponding to the cleavage site of PAR-2.**

Enzyme	K_m (μM)	k_{cat} (s^{-1})	k_{cat} / K_m (μM s^{-1})
Trypsin	5.5	86	15.6
RgpB	3.0	50	16.6

3.1.3 RgpB induces a calcium increase in human neutrophils

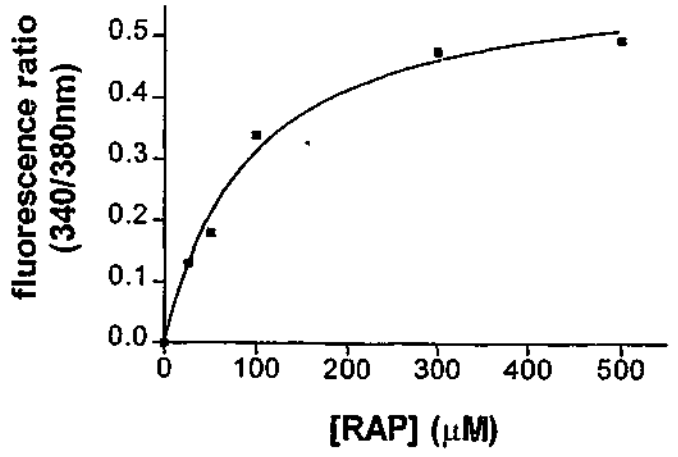
Neutrophils were isolated by fractionation of freshly drawn human blood from normal volunteers. As determined by Giemsa staining, neutrophil preparations contained more than 95% neutrophils, with eosinophils as the major contaminant. Neutrophils were loaded with Fura-2 and the ratio of the fluorescence at the two excitation wavelengths (340/380nm) was measured, which is proportional to the $[Ca^{2+}]_i$. Neutrophils from several donors were individually tested for a $[Ca^{2+}]_i$ response to RgpB, trypsin and the receptor agonist peptide (RAP) (Table 3.2.). All samples tested showed an increase in $[Ca^{2+}]_i$ in response to RAP treatment. The magnitude of the $[Ca^{2+}]_i$ response varied between individuals, confirming results described elsewhere (Howells *et al.*, 1997), where it was observed that the neutrophils of all donors express PAR-2, including some that did not respond to trypsin.

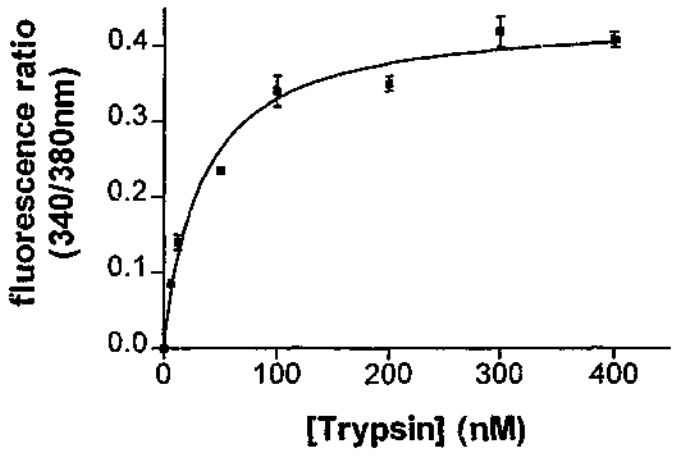
As with the RAP activation, the magnitude of the $[Ca^{2+}]_i$ increase caused by RgpB varied between donors, but the bacterial enzyme induced an increase in $[Ca^{2+}]_i$ only in neutrophils from donors that, in parallel, also exhibited a trypsin-induced increase in $[Ca^{2+}]_i$ [8 of the 12 donors] (Table 3.2). The variation in response and the lack of response to enzyme activation in certain donors is again in agreement with previous observations (Howells *et al.*, 1997). Neutrophils that did not respond to either protease did respond to the fMLP peptide, suggesting that G-protein-coupled receptor signalling pathways in otherwise non-responsive neutrophils were intact.

Table 3.2. **Intracellular calcium increase in response to various agonists in neutrophils from 12 donors**

	Fluorescence ratio 340/380 nm			
Donor	**RgpB** (200 nM)	**Trypsin** (200 nM)	**RAP** (300 μM)	**fMLP** (10 nM)
1	0.35	0.34	0.52	0.80
2	0.37	0.35	0.40	0.87
3	0.3	0.12	0.42	1.39
4	0.23	0.28	0.32	0.5
5	0.22	0.18	0.28	0.75
6	0.18	0.12	0.48	0.68
7	0.09	0.1	0.5	0.81
8	0.08	0.13	0.2	0.50
9	0	0	0.23	1.05
10	0	0	0.2	0.50
11	0	0	nd	0.30
12	0	0	nd	0.50

RgpB induced a dose-dependent increase in $[Ca^{2+}]_i$ in neutrophils. The concentration dependence of such an increase using neutrophils from a single donor is shown in Fig. 3.6, yielding an enzyme concentration inducing half the maximal response (EC_{50}) of 49.8 ± 3.9 nM, 39.2 ± 3.9 nM and 108 ± 10 μM for receptor activation by RgpB, trypsin and RAP, respectively.





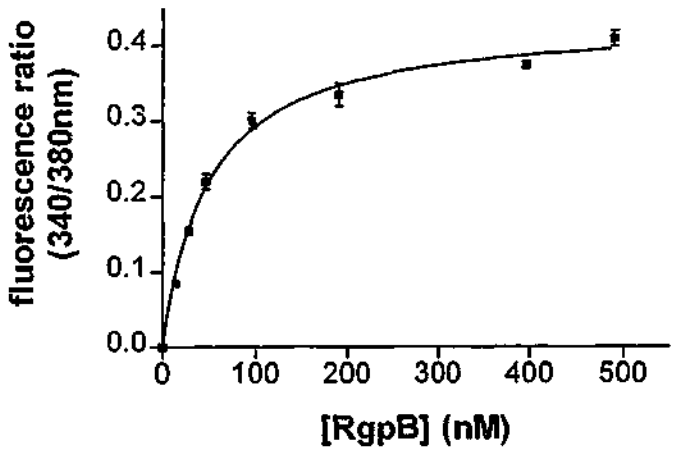


Figure 3.6. **The $[Ca^{2+}]_i$ response of neutrophils to different concentrations of RAP, trypsin or RgpB.**

Neutrophils (from donor 1) were loaded with Fura-2 and stimulated with RAP, trypsin or RgpB. The increase in $[Ca^{2+}]_i$ at different protease concentrations represents the mean ± SEM derived from three traces similar to those shown in Fig. 3.7 and Fig. 3.8.

Termination of PAR-2 signalling has been found to be mediated by protein kinase C (PKC), therefore acute activation of PKC decreases the calcium mobilisation in response to trypsin and RAP activation of PAR-2 (Bohm *et al.*, 1996a). The involvement of PKC in the RgpB-induced calcium increase in neutrophils was investigated using phorbol ester 12-myristate 13-acetate (PMA), a pharmacological activator of PKC (Castagna *et al.*, 1982). Pre-treatment of neutrophils from two donors with 60 nM PMA for 5 min resulted in a greatly diminished calcium response to trypsin (n=3), RAP (n=3) and RgpB (n=3). Pretreatment with dimethyl sulphoxide, the solvent in which the PMA was dissolved, did not affect the response to either trypsin (n=3), RAP (n=3) or RgpB (n=3) (Fig. 3.7). The effect of PMA on the calcium response to RgpB allows the suggestion that one or more PKC-dependent phosphorylations may be involved.

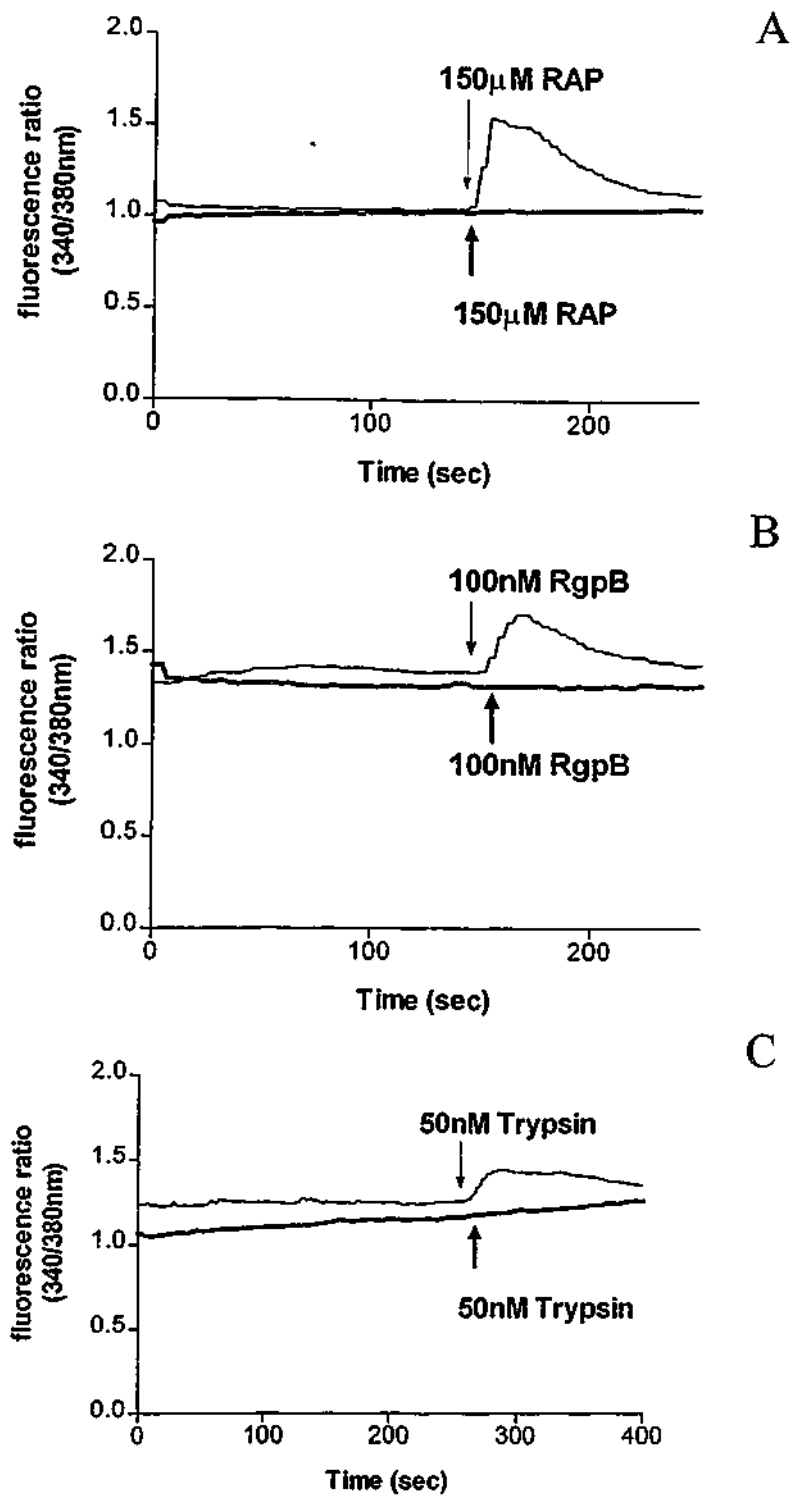


Figure 3.7. **Effects of pre-treatment with PMA on $[Ca^{2+}]_i$ responses in neutrophils.** Cells were pre-treated with 60 nM PMA (▬) or vehicle (—) for 5 min and then exposed to A) 150 μM RAP; B) 100 nM RgpB; C) 50 nM Trypsin. This figure shows the effect of PMA for one of the two donors in whom these effects were observed.

Protease-activated receptors undergo rapid desensitisation after a short period of activation by an enzyme agonist. Since exposure of cells to trypsin desensitises $[Ca^{2+}]_i$ responses to subsequent challenges with trypsin (Fig. 3.8A) due to cleavage of PAR-2 (Bohm *et al.*, 1996a), experiments were carried out using the $[Ca^{2+}]_i$ mobilisation assay in order to determine if RgpB and trypsin were activating the same receptor. When neutrophils were initially activated with RgpB, a secondary response to this enzyme was virtually eliminated (Fig. 3.8C). Exposure of neutrophils to trypsin also desensitised the $[Ca^{2+}]_i$ response to a second challenge with RgpB (Fig. 3.8B). Similarly, exposure of neutrophils to RgpB desensitised the response to a second challenge by trypsin (Fig. 3.8D). The fMLP peptide still caused an increase in $[Ca^{2+}]_i$ in RgpB treated neutrophils (Fig. 3.8C and D), indicating that depletion of intracellular calcium was not the mechanism of desensitisation. Since PAR-2 is a known protease-activated receptor on neutrophils which can be cleaved by trypsin (Howells *et al.*, 1997, Jenkins *et al.*, 1995), these results strongly suggest that trypsin and RgpB activate a common receptor on neutrophils, i.e. PAR-2. In certain cell types it has been found that treatment with a saturating concentration of RAP abolished a subsequent treatment with trypsin (Schechter *et al.*, 1998; Santulli *et al.*, 1995; Bohm *et al.*, 1996a) although in other cell types treatment with RAP did not affect a second response to trypsin (Bohm *et al.*, 1996a). In three donors, pre-treatment of neutrophils with 300 μM RAP caused a marked reduction in response to trypsin (Fig 3.9A, B) or RgpB (Fig. 3.9C, D). Pre-treatment of neutrophils from a fourth donor with a high concentration of RAP (500 μM) abolished a subsequent response to both trypsin and RgpB (Fig 3.9E, F, G), suggesting that the receptor activated by both trypsin and RgpB is PAR-2.

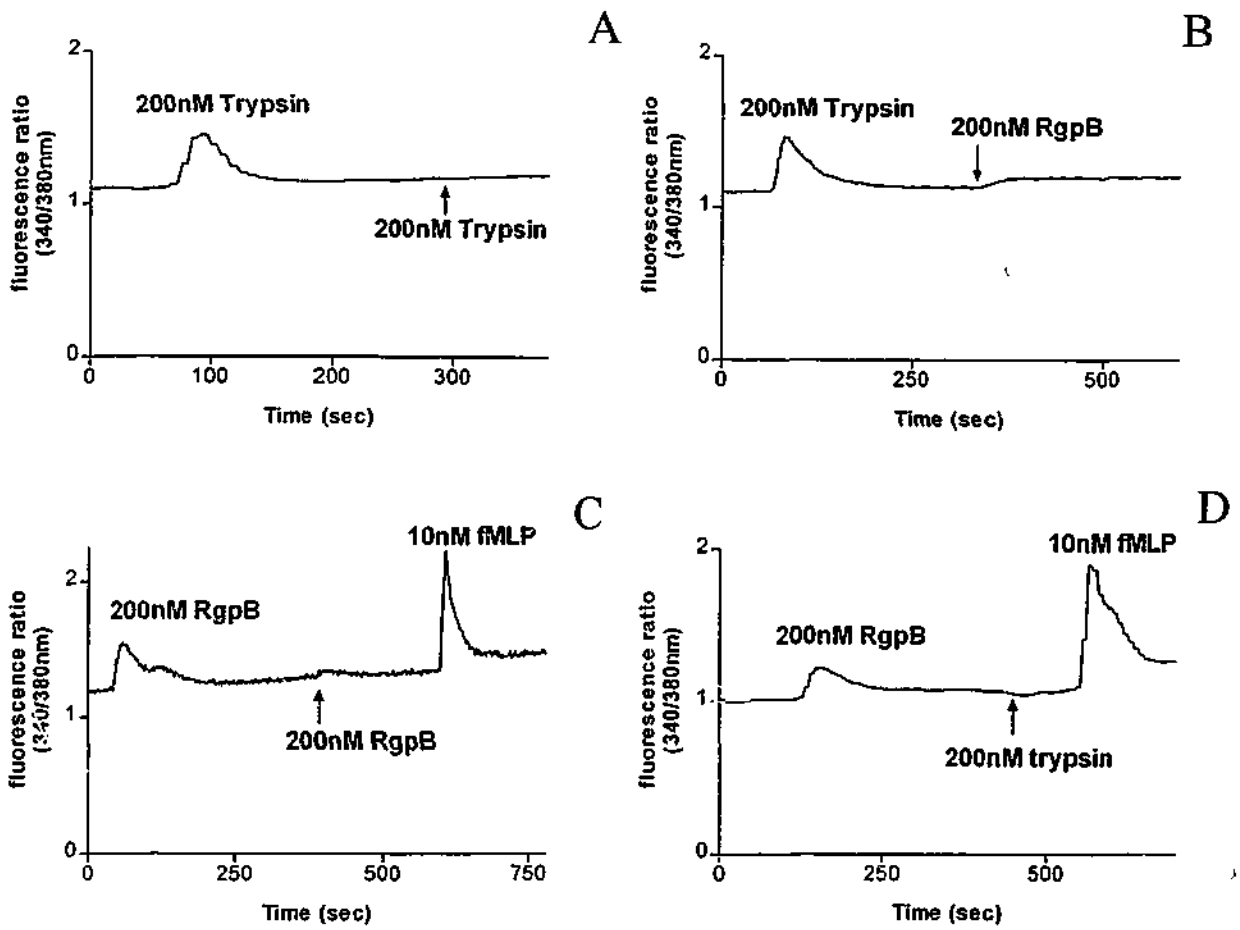


Figure 3.8. **Effects of desensitisation on $[Ca^{2+}]_i$ responses in neutrophils.**

Cells were exposed to A) 200 nM trypsin, then 200 nM trypsin; B) 200 nM trypsin, then 200 nM RgpB; C) 200 nM RgpB, then 200 nM RgpB, followed by 10 nM fMLP peptide; D) 200 nM RgpB, then 200 nM trypsin, followed by 10 nM fMLP peptide. This figure shows the desensitisation effects for one of the eight donors in whom these effects were observed.

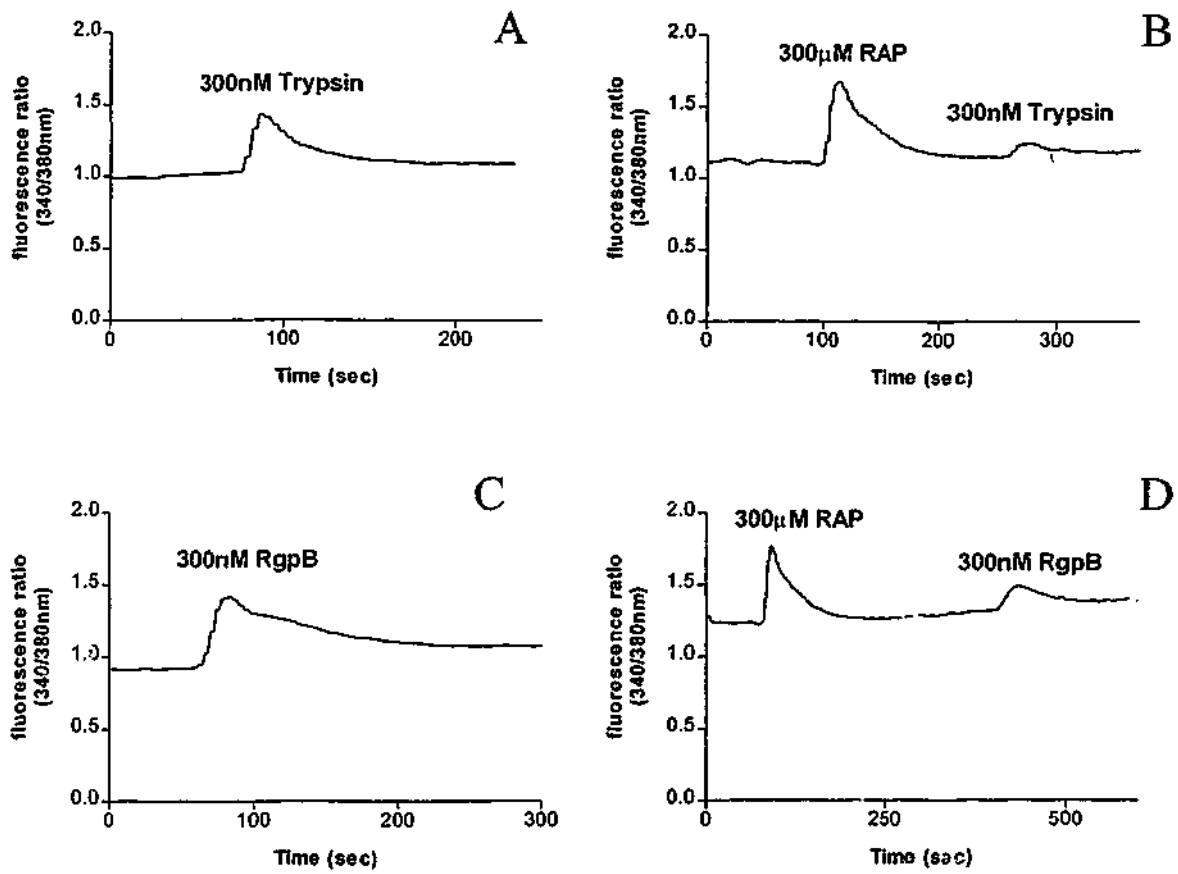


Figure 3.9. **Effects of desensitisation of $[Ca^{2+}]_i$ responses in neutrophils.**

Neutrophils from one donor were exposed to A) 300 nM Trypsin; B) 300 μM RAP, then 300 nM Trypsin; C) 300 nM RgpB; D) 300 μM RAP, followed by 300 nM RgpB. The results shown are also representative of those for a further two donors.

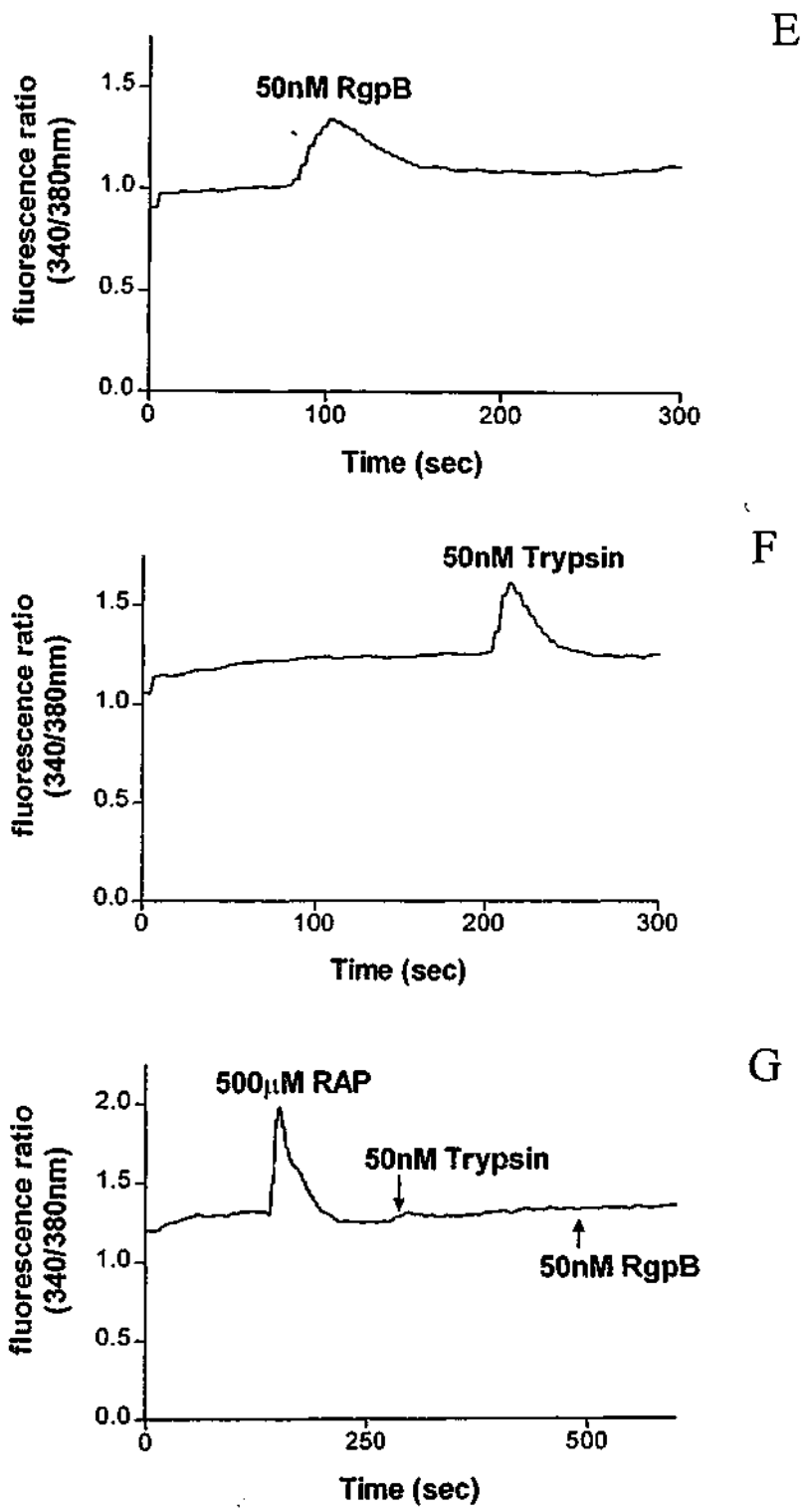


Figure 3.9. **Effects of desensitisation of $[Ca^{2+}]_i$ responses in neutrophils.**

Cells were exposed to A) 50 nM Trypsin; B) 50 nM RgpB; C) 600 μM RAP, then challenged with 50 nM Trypsin, followed by 50 nM RgpB. The results are representative of duplicate assays in neutrophils from this donor.

3.1.4 RgpB cleaves and activates human PAR-2 on CHO cells stably expressing human PAR-2

In order to verify that RgpB can cleave and activate PAR-2, the calcium response elicited by RgpB in transfected CHO cells stably expressing human PAR-2 (CHO PAR-2) in comparison to non-transfected CHO cells was investigated. Non-transfected CHO cells did not respond to either RAP or 1 nM trypsin (Fig. 3.10 A, C). Treatment of CHO PAR-2 cells with 100 μM RAP or 1 nM trypsin elicited an intracellular Ca^{2+} increase (Fig. 3.10 B, D). Non-transfected CHO cells did not respond to 1 nM RgpB (Fig. 3.10E), whereas treatment with 1 nM RgpB caused an intracellular Ca^{2+} increase in CHO PAR-2 cells (Fig. 3.10F), strongly indicating that PAR-2 is activated by RgpB.

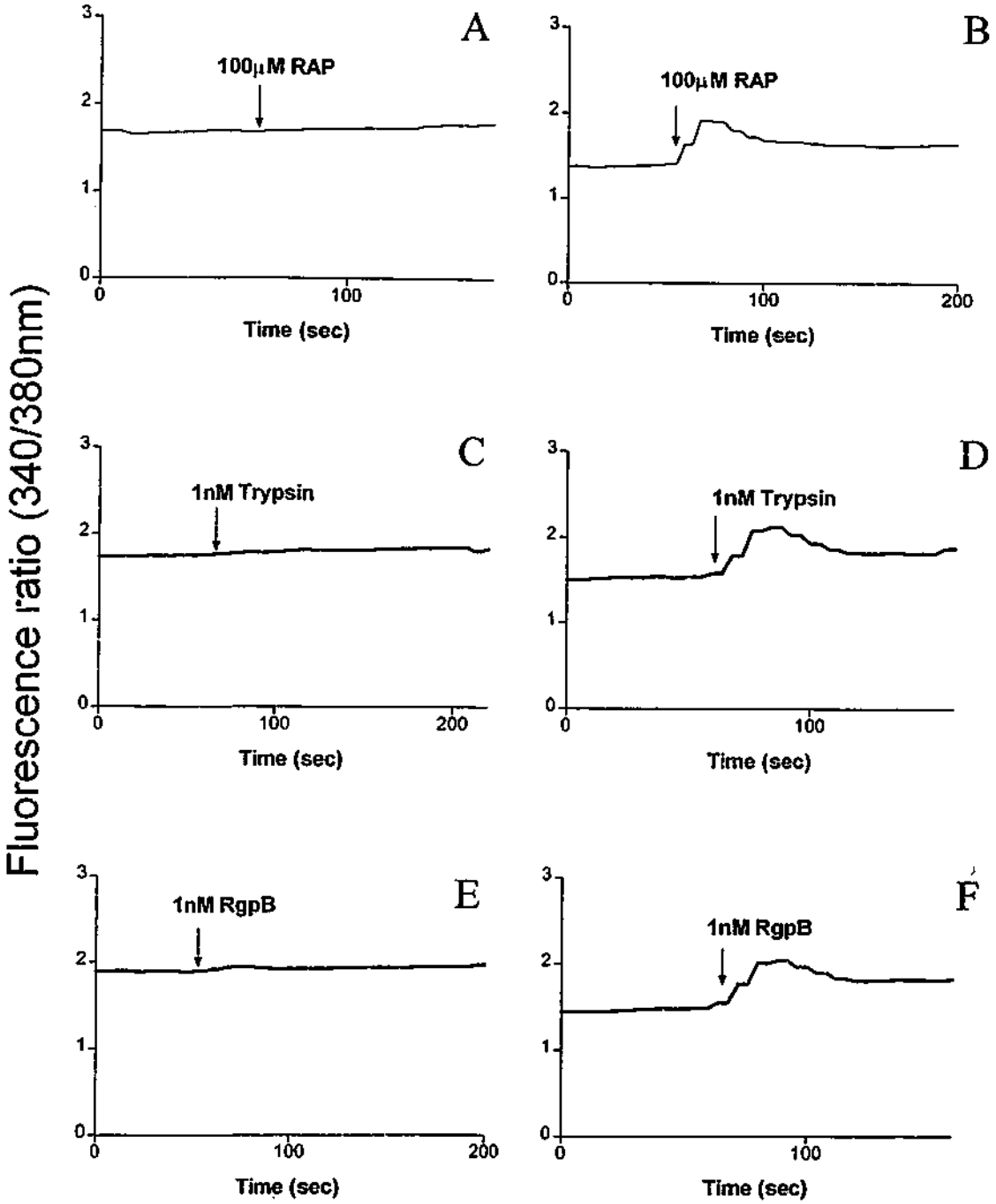


Figure 3.10. **R-gingipain (RgpB) activates PAR-2 on transfected cells stably expressing human PAR-2.**

Calcium mobilization in CHO cells (A, C, E) or CHO PAR-2 cells (B, D, F) induced by (A, B) 100 μM RAP; (C, D) 1 nM trypsin and (E, F) 1 nM RgpB. The traces are representative of three similar sets of data obtained.

3.1.5 Effect of PAR-2 activation on myeloperoxidase release from human neutrophils

Neutrophils possess granules containing enzymes that play an essential bactericidal role. Following phagocytosis of microorganisms by neutrophils, degranulation occurs as granules fuse with phagosomes containing the ingested particles and release their enzymes. Treatment of neutrophils with cytochalasin B "converts neutrophils from phagocytic cells into model secretory cells" (Smolen, 1989) as granules become fused with surface membranes and thus their granule contents can be studied extracellularly. Myeloperoxidase is an enzyme contained in the azurophilic granules, appears early in granulocytopoiesis and is essential for the oxygen-dependent bactericidal system of neutrophils (Klebanoff, 1971). An increase in $[Ca^{2+}]_i$ is thought to potentiate neutrophil degranulation, yet additional signals (possibly GTPgS) may be necessary for degranulation (Seetoo *et al.*, 1997; Niessen and Verhoeven, 1992; O'Flaherty *et al.*, 1991; Niessen *et al.*, 1991; Di Virgillo *et al.*, 1989). Agonists such as PMA cause degranulation without inducing a rise in intracellular calcium levels (Smolen, 1989; Di Virgillo *et al.*, 1989). To determine whether PAR-2 activation, which induces an intracellular calcium increase, can result in neutrophil degranulation, myeloperoxidase release from human neutrophils was examined. Isolated human neutrophils were incubated with cytochalasin B and then treated with various agonists. PMA was found to cause a large increase in myeloperoxidase release from human neutrophils, whereas neither RAP nor trypsin increased myeloperoxidase levels in any of the donors tested, indicating that PAR-2 activation does not induce neutrophil degranulation (Table 3.3). Similarly, RgpB also did not cause myeloperoxidase release in any of the donors tested (Table 3.3).

Table 3.3. **Myeloperoxidase release in response to various agonists in neutrophils [a].**

ΔA_{450}/min

Donor	Control	PMA 60 nM	Trypsin 150 nM	RAP 300 μM	RgpB 150 nM
1	4.5, 3.9	6.4, 5.7	4.1, 4.1	4.2, 4.6	4.5, 4
2	2.5, 3.2	4.3, 4.8	2.6, 2.3	2.3, 3.1	2.6, 3.0
3	4.8, 4.9	8.6, 9.3	3.9, 4.6	4.3, 4.7	4.2, 4.9
4	29.3, 33	97.6, 99	nd	27.7, 30.6	nd
5	11, 8.9	27, 28	nd	nd	12, 7

[a] Results are presented as ΔA_{450}/min values for duplicate wells.

3.1.6 Up-regulation in the expression of CD11b on human

Activation of PAR-2 on neutrophils by RAP has been reported to increase expression of CD11b (Howells *et al.*, 1997). CD11b levels were measured using whole blood (Howells *et al.*, 1997), as it has been shown that neutrophil isolation procedures cause neutrophil priming and adhesion molecules on their surface are subject to fixation artefacts (Macey *et al.*, 1992). Howells *et al.* (1997) used blood anticoagulated with heparin to avoid changes in plasma calcium levels (McCarthy and Macey, 1996). In this study, CD11b expression was also examined in whole human blood from healthy volunteers, anticoagulated with heparin, which was treated with various agonists for 15 min and the median fluorescence intensity was measured (Table 3.4). Blood from some donors was treated with only one agonist in an attempt to reduce handling and time delays before analysis. Treatment with RAP lead to an increase in CD11b expression, although RAP appears to be a weaker agonist compared to fMLP (Table 3.4). There was a marked variation in the magnitude of response of neutrophils

between donors 1 and 2, both to RAP and fMLP, which is in agreement with previous observations (Howells *et al.*, 1997). RgpB was also found to cause an increase in CD11b expression, although the magnitude of increase was smaller compared to the increase induced by RAP in donor 2.

Table 3.4. **CD11b increase in response to various agonists in neutrophils**

	CD11b Median Fluorescence intensity				
Donor	**Control** cysteine buffer	**RgpB** 50 nM	**Control**	**RAP** 300 μM	**fMLP** 100 nM
1	nd	nd	108, 117	289, 327	697, 673
2	107, 100	237, 200	171, 159	486, 414	1333, 1000
3	13,13	29, 21	nd	nd	nd
4	62, 67	264, 171	110	nd	352, 327
5	16, 13	35, 25	14, 15	nd	25

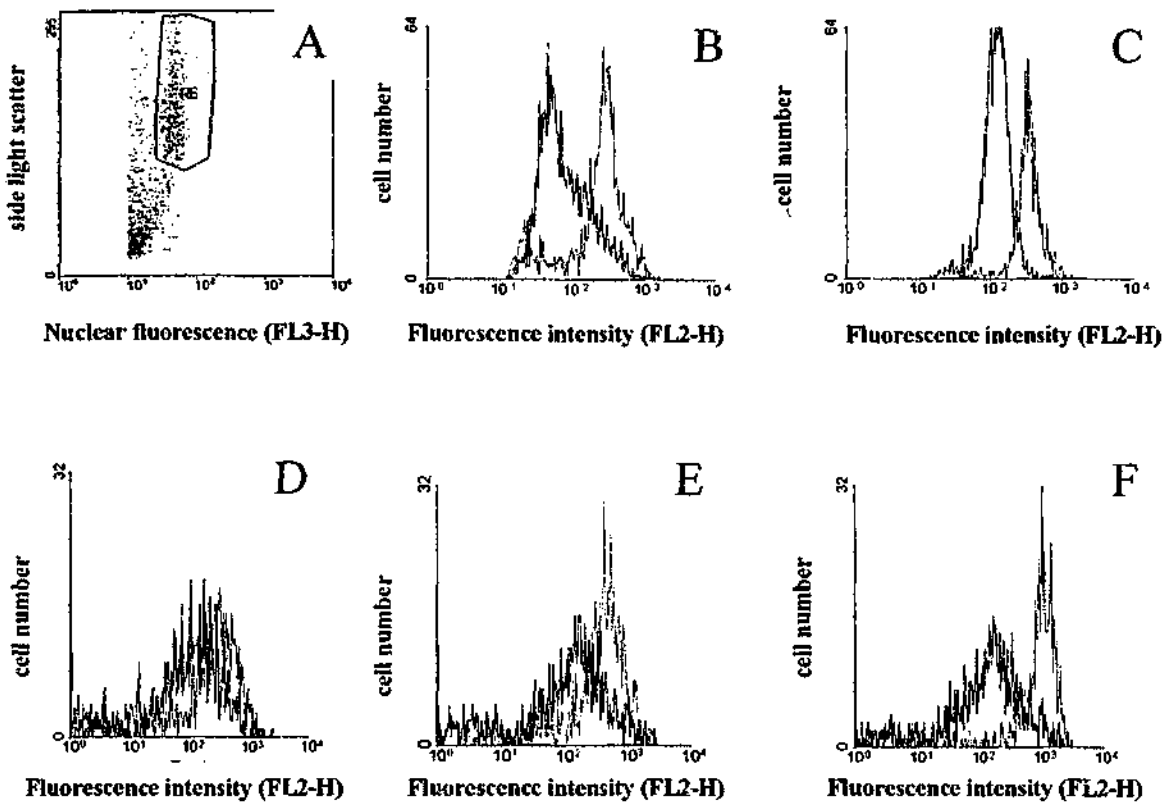


Figure 3.10. **Flow cytometric analysis of CD11b in peripheral blood neutrophils.**
Scatter plot showing nuclear fluorescence intensity on the *x* axis and side light scatter on the *y* axis (A). Granulocytes are gated in whole blood preparations as shown.
Blood was stained with CD11b antibodies and the fluorescence intensity is shown on the *x* axis. CD11b staining is shown for donor 4 (B&C) and donor 2 (D-F). The peak on the left (black) in each instance represents the control treatment. The peak on the right represents treatment with the individual agonists, RgpB (red), RAP (green) and fMLP (blue).

3.2 Discussion

Human neutrophils have been shown to express protease activated receptor-2 (PAR-2) and stimulation of the receptor was shown to lead to neutrophil activation (Howells *et al.*, 1997; Vergnolle, 1999). In this study we investigated whether the protease RgpB from the bacterium *P. gingivalis* can activate PAR-2 on neutrophils. A synthetic peptide corresponding to part of the extracellular domain of PAR-2 was cleaved by trypsin and RgpB with similar kinetic constants, indicating that RgpB could possibly activate the receptor.

RgpB stimulated an intracellular Ca^{2+} mobilisation in neutrophils with similar efficiency and potency to trypsin. Calcium mobilisation varied between donors, but neutrophils from donors responding to trypsin also responded to RgpB, whereas neutrophils that did not exhibit Ca^{2+} mobilisation in response to trypsin also did not respond to RgpB. Neutrophils from all donors tested, even those that did not respond to trypsin or RgpB, did respond to RAP. One possible explanation is that PAR-2 is already cleaved on the surface of these cells and therefore can no longer be activated by a protease, but still responds to its agonist peptide. It has been shown that following activation of PAR-1 a small of the portion of the internalised receptors are recycled to the cell surface, and respond to the agonist peptide TRAP but cannot be activated by thrombin (Hoxie *et al.*, 1993; Brass, 1992; Brass et al., 1994). Dery *et al.* (1999) have suggested that some of the activated PAR-2 molecules may also recycle to the cell surface. Thus, the response to RAP in those neutrophils that do not respond to proteases may be due to the presence of recycled PAR-2 molecules on their surface.

Acute activation of PKC, in transfected kidney epithelial cells stably expressing PAR-2 and intestinal epithelial cells naturally expressing PAR-2, has previously been shown to decrease the calcium mobilisation in response to trypsin and RAP activation of PAR-2 (Bohm *et al.*, 1996a). Activation of PKC by PMA led to inhibition of

calcium response in neutrophils in response to both RAP and trypsin. Similarly, PMA blocked the calcium increase in response to RgpB. PMA activation of PKC terminates PAR-2 signalling and it has been suggested that PKC may directly phosphorylate PAR-2 (Bohm *et al.*, 1996a). PMA can also block the release of calcium from intracellular stores, by inhibiting the inositol triphosphate (IP_3) production pathway through its effect on phospholipase C (PLC; Smith *et al.*, 1987; Watson and Lapetina, 1985). Protein kinase C can phosphorylate PLCβ (Ryu *et al.*, 1990) and the α subunits of certain G-proteins (Bushfield *et al.*, 1990; Carlson *et al.*, 1989). The effect of PMA on the calcium response to RgpB allows the suggestion that one or more PKC-dependent phosphorylations may be involved.

Protease-activated receptors undergo rapid desensitisation after a short period of activation by an enzyme agonist. Exposure of cells to trypsin desensitized $[Ca^{2+}]_i$ responses to subsequent challenges with trypsin. Similarly, when neutrophils were initially activated with RgpB, a secondary response to this enzyme was virtually eliminated. The Ca^{2+} response to RgpB in neutrophils was abolished by prior treatment with trypsin. Similarly, exposure of neutrophils to RgpB desensitises the Ca^{2+} response to a second challenge with trypsin. Since PAR-2 is the only known protease-activated receptor on neutrophils which can be cleaved by trypsin (Howells *et al.*, 1997, Jenkins *et al.*, 1995), these results strongly suggest that trypsin and RgpB activate a common receptor on neutrophils, i.e. PAR-2. It has been reported that in transfected kidney epithelial cells stably expressing PAR-2 treatment with RAP reduced a subsequent response to RAP and did not affect a second response to trypsin (Bohm *et al.*, 1996a). Yet, in keratinocytes and epithelial cells naturally expressing PAR-2, treatment with a saturating concentration of RAP abolished the response to secondary treatment with RAP and markedly reduced or abolished a subsequent treatment with trypsin (Bohm *et al.*, 1996a; Schechter *et al.*, 1998; Santulli *et al.*, 1995).

The difference in the desensitising effect of RAP to subsequent treatment with RAP or trypsin may be due to differences in the number of receptors expressed on the surface of the cells examined. In the present study it was found that pre-treatment of neutrophils with a high concentration of RAP abolished (in one donor) and substantially reduced (in three others) a subsequent response to both trypsin and RgpB, suggesting that the receptor activated by both trypsin and RgpB was PAR-2. The ability of RgpB to activate PAR-2 was verified by the finding that RgpB induced an increase in intracellular calcium in transfected CHO cells stably expressing PAR-2, but did not elicit a response in non-transfected CHO cells.

In a previous study, a variation in response to trypsin and RAP in neutrophils from different donors was reported (Howells *et al.*, 1997). This variation did not correlate with the levels of expression of PAR-2 between the different donors as determined by FACS analysis (Howells *et al.*, 1997). In the present study, Ca^{2+} mobilisation in response to trypsin, RgpB and RAP was also found to vary between donors. The handling of blood leads to partial priming of neutrophils (Macey *et al.*, 1992), thus the variation between donors may in part be due to the differences in the extent of priming of the neutrophils between donors. Priming is a process by which very low concentrations of agonists, which do not cause neutrophil activation, cause an up-regulation of the response of neutrophils to a subsequent stimulation (Edwards *et al.*, 1995; Walker and Ward, 1992).

Intracellular calcium is an important second messenger that plays a role in activation of neutrophil functions such as degranulation and chemotaxis (Di Virgillo *et al.*, 1989). Since PAR-2 activation by RAP, trypsin or RgpB leads to intracellular calcium increase, possible subsequent cellular responses were investigated. It has been shown that stimulation of PAR-2 by RAP leads to neutrophil activation as indicated by an increase in the expression of CD11b (Howells *et al.*, 1997). CD11b, a subunit of the

neutrophil adherence receptor, is a calcium-dependent epitope in human neutrophils, that is rapidly expressed on the cell surface following activation (Hickstein *et al.*, 1989). Howells *et al.* (1997) observed variability between donors, as the CD11b expression of the neutrophils from two of the five donors tested did not increase in response to RAP. In the current study, treatment of neutrophils with RAP or RgpB from *P. gingivalis* was found to cause an increase in CD11b expression, although RgpB was found to be a weaker agonist. The results of PAR-2 activation on neutrophils by RgpB in whole blood should be interpreted with caution, due to the possible effect of RgpB on other cells and proteins in blood (Travis and Potempa, 2000). Similarly, Howells *et al.* (1997) avoided examining the effect of trypsin on CD11b expression in neutrophils in whole blood. It is possible that the concentration of RgpB necessary to cause CD11b up-regulation on neutrophils in whole blood may be higher than that needed to induce calcium mobilization in isolated neutrophils and thus other RgpB concentrations should be examined. Interestingly, lipopolysaccharide from *P. gingivalis* has also been found to enhance expression of CD11b and Mac-1 receptor on the surface of human neutrophils (Wilton *et al.*, 1990). There was a marked variation in the magnitude of response of neutrophils between the donors, both to RAP and fMLP, which is in agreement with previous observations (Howells *et al.*, 1997) and may be due to different priming of the neutrophils between donors. Activation of PAR-2 and subsequent up-regulation of CD11b, a subunit of the adherence receptor, can therefore lead to neutrophil adherence and migration into inflamed tissue. Neutrophil activation by RgpB acting via PAR-2 may thus promote the observed accumulation of neutrophils into the infected gingiva and thereby exacerbate the inflammation seen in periodontal disease. Degranulation of neutrophils following PAR-2 activation was also examined. It was found that PAR-2 activation on human neutrophils by RAP or trypsin did not induce myeloperoxidase release in any of the donors tested, indicating that this receptor

is probably not responsible for degranulation of azurophilic granules. Similarly, RgpB also did not enhance myeloperoxidase release. Lack of degranulation following exposure of neutrophils to *P. gingivalis* and its products may account for the persistence of the bacterium in the periodontal pocket. Overall, in periodontal disease there seems to be an accumulation of neutrophils in periodontal pockets (Murray and Patters, 1980), but little or no actual phagocytosis of bacterial pathogens (Schenkein, 1999).

In the present study, it was observed that relatively low concentrations of RgpB, in the nanomolar range, instantly induce intracellular calcium mobilisation following activation of PAR-2 on neutrophils. Sugawara *et al.* (2000) showed that treatment of monocytes with 1 μM RgpB for 30 min caused proteolysis of CD14, which functions as a major receptor for LPS (Wright *et al.*, 1990) and reduced the response to LPS. In contrast the expression of Toll-like receptor 4, CD18, CD54 (ICAM-1) and CD59 were increased following treatment with gingipains-R. Neutrophils also express CD14 (Haziot *et al.*, 1993) which may be affected by RgpB, therefore it is necessary to examine the expression of various cell surface molecules in order to evaluate the overall effect of RgpB on neutrophils. There is growing evidence that neutrophils can produce a number of cytokines such as Interleukin-1α (IL-1α), IL-8, IL-12 and Tumor Necrosis Factor-α (Cassatella *et al.*, 1999). A wide range of stimuli can cause expression of cytokines by neutrophils, including activation of the G-protein-coupled receptors for fMLP and C5a (Cassatella *et al.*, 1999). PAR-2 activation in keratinocytes has also been shown to induce IL-6 and IL-8 secretion (Wakita *et al.*, 1997; Hou *et al.*, 1998), therefore it would be of interest to examine whether PAR-2 activation by RgpB on neutrophils can affect cytokine production. The finding that RgpB can activate PAR-2 on human neutrophils demonstrates that *P. gingivalis*, through its products, can actually interact with host cell receptors to induce intracellular signalling and possibly contribute to the inflammatory process.

CHAPTER 4

RgpB from *Porphyromonas gingivalis* Activates PAR-1 and PAR-2 on Human Oral Epidermoid cells and Induces Interleukin-6 secretion

4.0 Introduction

In periodontal disease, bacteria bind to the tooth surface, extend into the gingival sulcus and subsequently form subgingival plaques in the periodontal pocket (Fig. 1.6). At the base of the gingival sulcus, the gingival epithelium forms a thin epithelial lining termed the junctional epithelium, which is directly attached to the tooth and is a specialised layer of non-keratinised cells. A periodontal pocket is an abnormal deepening and enlargement of the gingival sulcus that occurs during the progression of periodontitis when the plaque bacteria colonise and disrupt the attachment between the junctional epithelium and the tooth.

Thus the cells of the junctional epithelium form an interface with the subgingival bacteria and are directly exposed to the bacteria and their products. The interaction that occurs between the periodontal bacteria and epithelial cells and the subsequent molecular signals that are generated, are of great interest as they may contribute to the overall effect of the bacteria on the host and the progression of periodontal disease. The epithelial cells represent a cell barrier limiting the spread of bacteria within the periodontal pocket. However, the periodontal pathogen *P. gingivalis* has been detected in gingival tissues *in vivo* (Saglie *et al.*, 1988), indicating that bacteria may pass through

this epithelial cell barrier. Certain strains of *P. gingivalis* have also been found to invade epithelial cells *in vitro* (Sandros *et al.*, 1993; Lamont *et al.*, 1995) and even replicate within these cells *in vitro* (Madianos *et al.*, 1996).

Epithelial cells may contribute to the host's defence as a number of studies have shown that gastrointestinal and uroepithelial cells express pro-inflammatory cytokines following exposure to invasive and non-invasive bacteria (Agace *et al.*, 1993; Eckmann *et al.*, 1993; Jung *et al.*, 1995; Rasmussen *et al.*, 1997). Periodontal bacteria and their products can also elicit signals in epithelial cells and produce a cellular response. Bacteria common in the normal oral flora such as *Fusobaterium nucleatum, Eikenella corrodens* and *Actinobacillus actinomycetemcomitans* were found to induce the production of IL-8 in gingival epithelial cells (Yumoto *et al.*, 1999; Han *et al.*, 2000; Huang *et al.*, 1998). *P. gingivalis* was found to inhibit IL-8 accumulation (Huang *et al.*, 1998; Darveau *et al.*, 1998), and it was determined that IL-8 down-regulation was dependent upon invasion of the epithelial cells by the bacterium (Darveau *et al.*, 1998). Invasion efficiency of *P. gingivalis* is relatively low and immunohistochemical studies have shown that the bacterial cells are in close contact with the epithelium in the periodontal pockets (Noiri et al., 1997), therefore it is of interest to examine whether this bacterium can affect epithelial cells without being inside the cells.

In order to understand whether a "molecular dialogue" between epithelial cells and *P. gingivalis* can occur, the bacterial products affecting extracellular components of the cells and thereby eliciting an intracellular signal leading to a cellular response need to be identified. One of the bacterium's products are gingipains which are not only associated with blebs of the outer membranes of *P. gingivalis*, but are also released into the medium in a soluble form and have been detected in gingival crevicular fluid, a plasma exudate released in the periodontal pocket (Wikstrom *et al.*, 1994). The arginine-specific gingipain, RgpB, has been found to cleave and activate PAR- 2 on

human neutrophils and on transfected cells stably expressing human PAR-2 (Chapter 3). Gingival fibroblasts express PAR-1 and activation of this receptor stimulated IL-6 secretion (Hou *et al.*, 1998b). Human keratinocytes were found to express PAR-1 and PAR-2 and their activation induced IL-6, IL-8 and granulocyte-macrophage colony stimulating factor secretion (Wakita *et al.*, 1997; Hou *et al.*, 1998b), indicating that proteases, acting through these receptors, can affect the course of physiological and pathological processes by stimulating the production of such pro-inflammatory cytokines. KB-60 is an oral epithelial cell line that has been extensively used as a model to study gingival epithelial cells. The aim of this study is to determine whether KB-60 cells express protease-activated receptors, investigate whether RgpB can cleave and activate these receptors resulting in the induction of an intracellular signal, and whether the cellular response induced affects the secretion of cytokines by these cells.

4.1 Results

4.1.1 KB-60 cells express PAR-1, PAR-2 and PAR-3

Total RNA from KB-60 cells was isolated and transcribed into the cDNA. The cDNA was amplified by specific primers and revealed the presence of PAR-1, PAR-2 and PAR-3 mRNA; no expression of PAR-4 was detected (Fig. 4.1A &B). Primers for PAR-1 did not span an intron thus RNA isolated from platelets, known to express PAR-1, was used as a positive control to verify the expression of PAR-1 in KB-60 cells. PAR-1 expression was also confirmed by the fact that only RNA from KB-60 cells that had undergone reverse transcription resulted in the appearance of the expected PAR-1 cDNA product. (Fig. 4.1B). Although competitive RT-PCR would be required in order to determine the quantity of each PAR mRNA in KB-60 cells, based on the intensity of the PAR-2 product it appears that PAR-2 is expressed at a higher level than PAR-1 and PAR-3.

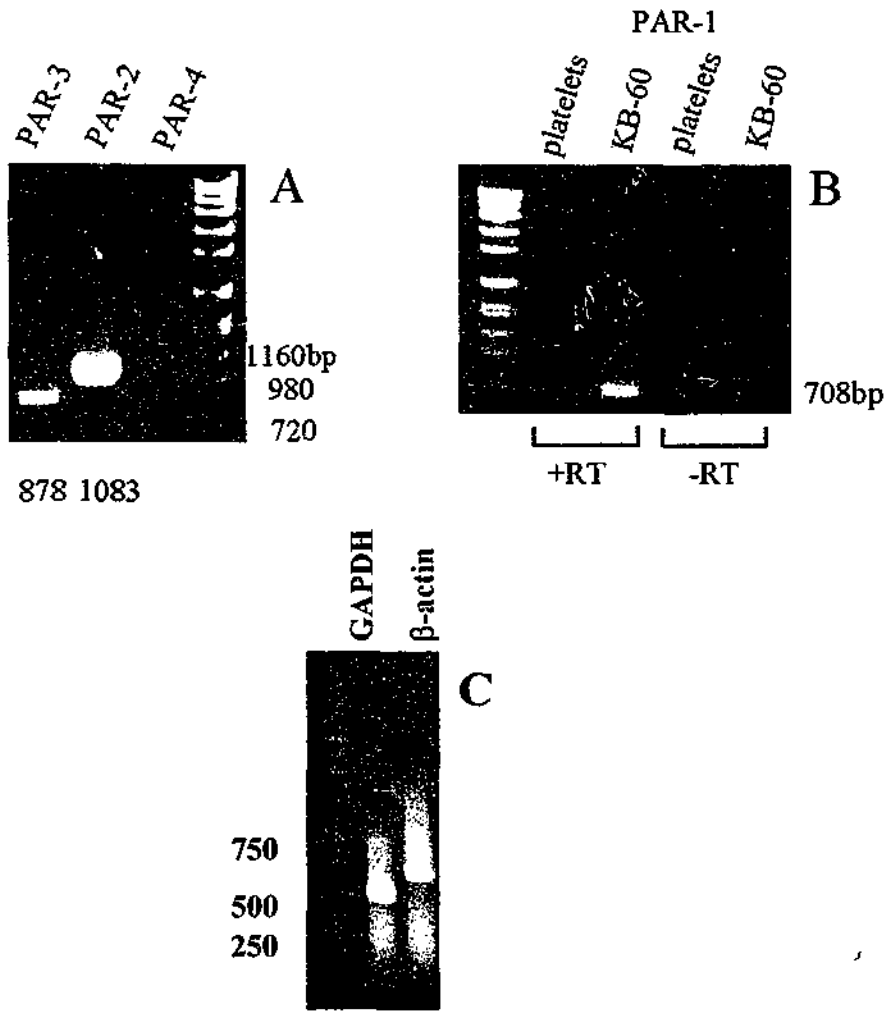
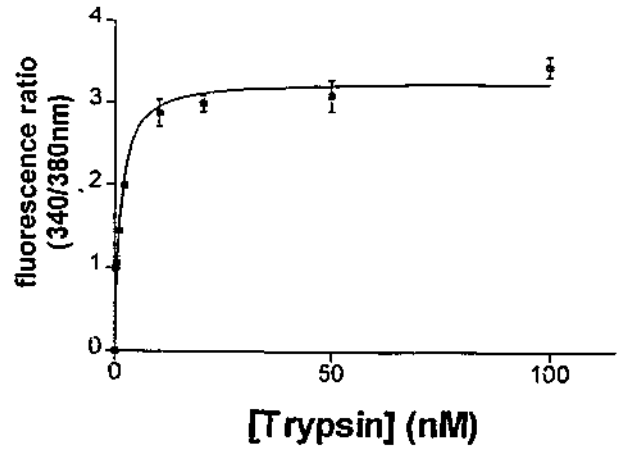


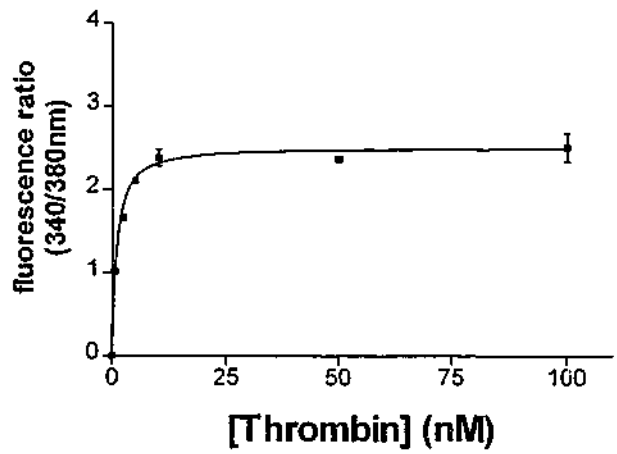
Figure 4.1. **Expression of mRNAs encoding protease-activated receptors in KB60 cells.**
RNA isolated from KB60 cells or platelets, was analysed for PAR transcripts by RT-PCR as described in the Materials and Methods. RNA from platelets was used as a positive control for expression of PAR-1. A) KB60 cells express PAR-2 (expected size 1083bp) and PAR-3 (878bp); B) KB60 cells and platelets express PAR-1 (708bp); C)GAPDH and β-actin expression in KB60 cells.

4.1.2 RgpB increased intracellular calcium levels $[Ca^{2+}]_i$ in KB-60 cells

KB-60 cells were tested for a $[Ca^{2+}]_i$ response to trypsin, thrombin, and RgpB and all proteases were found to induce a concentration-dependent increase in $[Ca^{2+}]_i$ (Fig. 4.2). These data yield an enzyme concentration inducing the half maximal response (EC_{50}) of 0.94 nM, 1.77 nM and 5.52 nM for thrombin, trypsin and RgpB, respectively. Trypsin and RgpB appear to elicit a higher maximal mobilisation of calcium compared to thrombin (Fig. 4.2).

Thrombin is a known activator of PAR-1, PAR-3 and PAR-4 (Vu *et al.*, 1991a; Ishihara *et al.*, 1997; Xu *et al.*, 1998) and trypsin can activate PAR-1, PAR-2 and PAR-4 (Vu *et al.*, 1991a; Vouret-Craviari *et al.*, 1995; Parry *et al.*, 1996; Nystedt *et al.*, 1994; Molino *et al.*, 1997a; Xu *et al.*, 1998). KB-60 cells were found to express PAR-1, PAR-2 and PAR-3; thus cleavage and activation of these receptors probably mediates the calcium mobilisation induced by trypsin and thrombin. In support of this hypothesis is the finding that treatment of KB-60 cells with RAP or TRAP (Fig. 4.3A & B), but not TRAP-4 (Fig. 4.3C), caused an increase in $[Ca^{2+}]_i$, verifying the expression of PAR-1 and PAR-2 on KB-60 cells. RgpB which had been inactivated by antipain did not induce a calcium response in KB-60 cells (Fig. 4.3D), indicating that the $[Ca^{2+}]_i$ increase induced is due to the proteolytic activity of the enzyme.





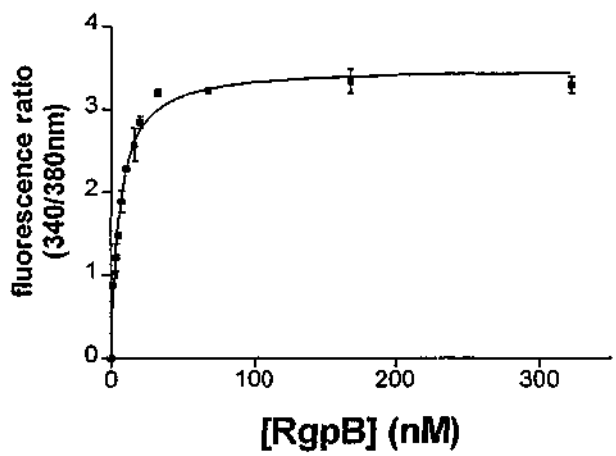


Figure 4.2. **The $[Ca^{2+}]_i$ response of KB60 cells to different concentrations of trypsin, thrombin and RgpB.**

Each data point represents the mean from two traces similar to those shown in Fig. 4.3.

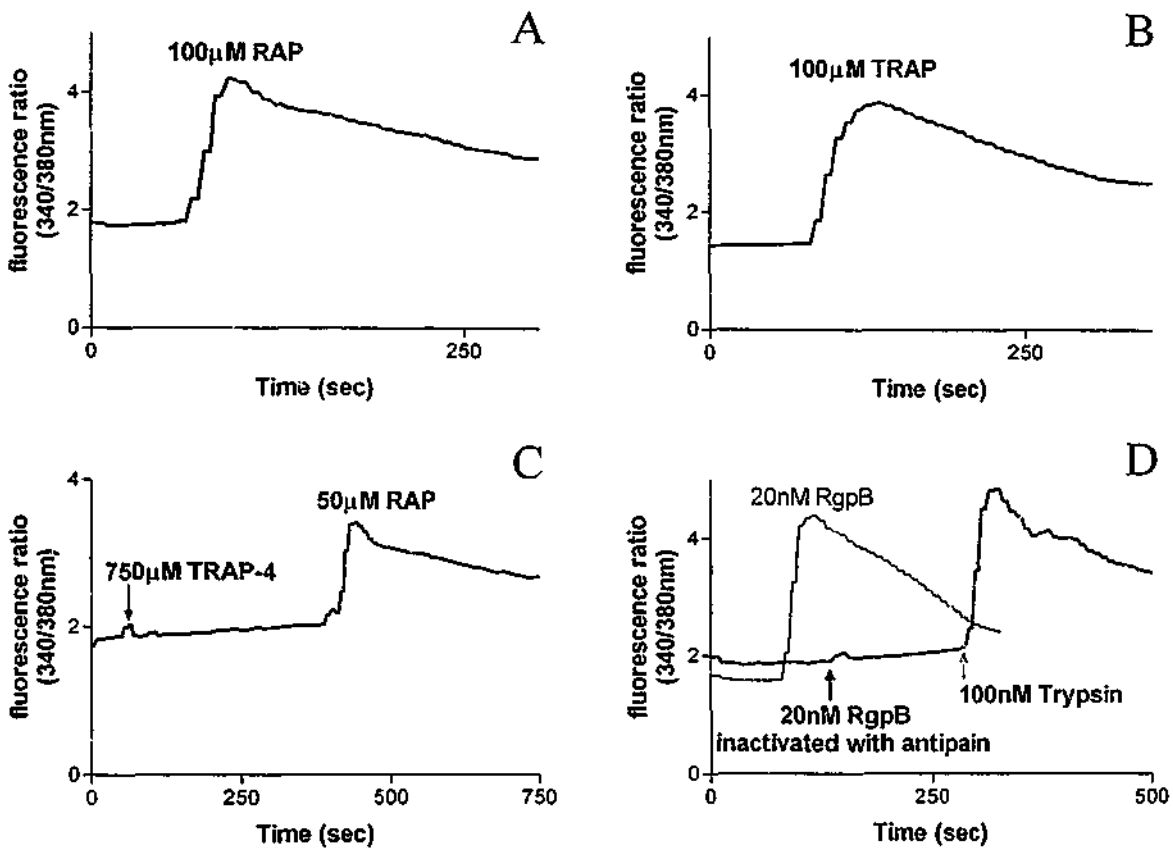


Figure 4.3. **$[Ca^{2+}]_i$ mobilisation in KB60 cells in response to protease-activated receptor agonists and following treatment with RgpB.**

The $[Ca^{2+}]_i$ responses in KB60 cells to: A) 100 μM RAP; B) 100 μM TRAP; C) 750 μM TRAP-4; D) 20 nM RgpB inactivated with antipain (⟶), followed by trypsin to demonstrate that the cells were still capable of mobilising $[Ca^{2+}]_i$; or 20 nM RgpB alone (—).

The question then addressed was whether the increase in $[Ca^{2+}]_i$ induced by RgpB activity is due to cleavage of a protease-activated receptor (PAR) on the surface of KB-60 cells. Once a PAR is cleaved by a protease it is unable to be activated a second time by the same or another protease in a short period of time. As cleavage of a PAR desensitizes $[Ca^{2+}]_i$ responses to subsequent protease challenges, desensitization of $[Ca^{2+}]_i$ responses by trypsin, thrombin and RgpB were examined. Desensitizations between thrombin and trypsin, which are known PAR activators were firstly examined. As seen in Fig. 4.2, 100 nM trypsin can elicit a maximal calcium mobilisation, whereas 10 nM trypsin induces a sub-maximal increase in intracellular calcium levels. The same observation was made for 100 nM and 10 nM thrombin. RgpB at a concentration of 10 nM induces a sub-maximal increase and at 20 nM elicits a response very close to the maximal, and due to limited stocks of the enzyme this concentration was used for maximal calcium mobilization in the desensitisation studies. When inducing a maximal calcium increase the protease cleaves the majority of available protease-activated receptors that can be activated by the given protease.

Trypsin would be expected to activate PAR-1 and PAR-2 on the surface of KB60 cells, while thrombin would activate PAR-1 and PAR-3. Therefore, pre-treatment of KB-60 cells with trypsin would cause cleavage and desensitization of PAR-1 and PAR-2, whereas PAR-3 could still be activated by thrombin. Exposure of the cells to thrombin would cause activation and desensitization of PAR-1 and PAR-3, leaving only PAR-2 available for activation by trypsin. Therefore it should be evident that treatment of KB60 cells with trypsin followed by thrombin would yield information on the contribution that PAR-3 makes to thrombin-induced calcium signalling in these cells. Treatment of cells with thrombin followed by trypsin should provide information on the contribution of PAR-2 to the trypsin-induced calcium signalling. It was found that pre-treatment of KB-60 cells with 10 nM trypsin reduced a secondary response to

10 nM thrombin by approximately 50% (Fig. 4.4A), whereas exposure of cells to 10 nM thrombin had no apparent effect on a subsequent challenge with 10 nM trypsin (Fig. 4.4B). In each case cells could still respond to ADP, indicating that the cells were capable of responding to other agonists. Treatment of cells with 100 nM trypsin completely abolished a subsequent response to 10 nM thrombin (Fig. 4.4C), while treatment of cells with 100 nM thrombin reduced a secondary response to 10 nM trypsin by 20% (Fig. 4.4D) without affecting a subsequent response to ADP. Treatment of KB-60 cells with a maximal trypsin concentration reduced a subsequent response to a maximal concentration of thrombin by approximately 95% (Fig. 4.4E), whereas exposure of KB-60 cells to a maximal concentration of thrombin reduced a secondary response to a maximal concentration of trypsin by approximately 20% (Fig. 4.4F). The fact that following treatment with trypsin the residual response to thrombin was minimal suggests that PAR-3 mediated thrombin signalling represents a minimal portion, if any of the thrombin–induced calcium mobilization. The finding that subsequent to treatment with thrombin the response to trypsin was reduced only by a small amount, suggests that PAR-2 mediates the majority of the trypsin-induced calcium signalling.

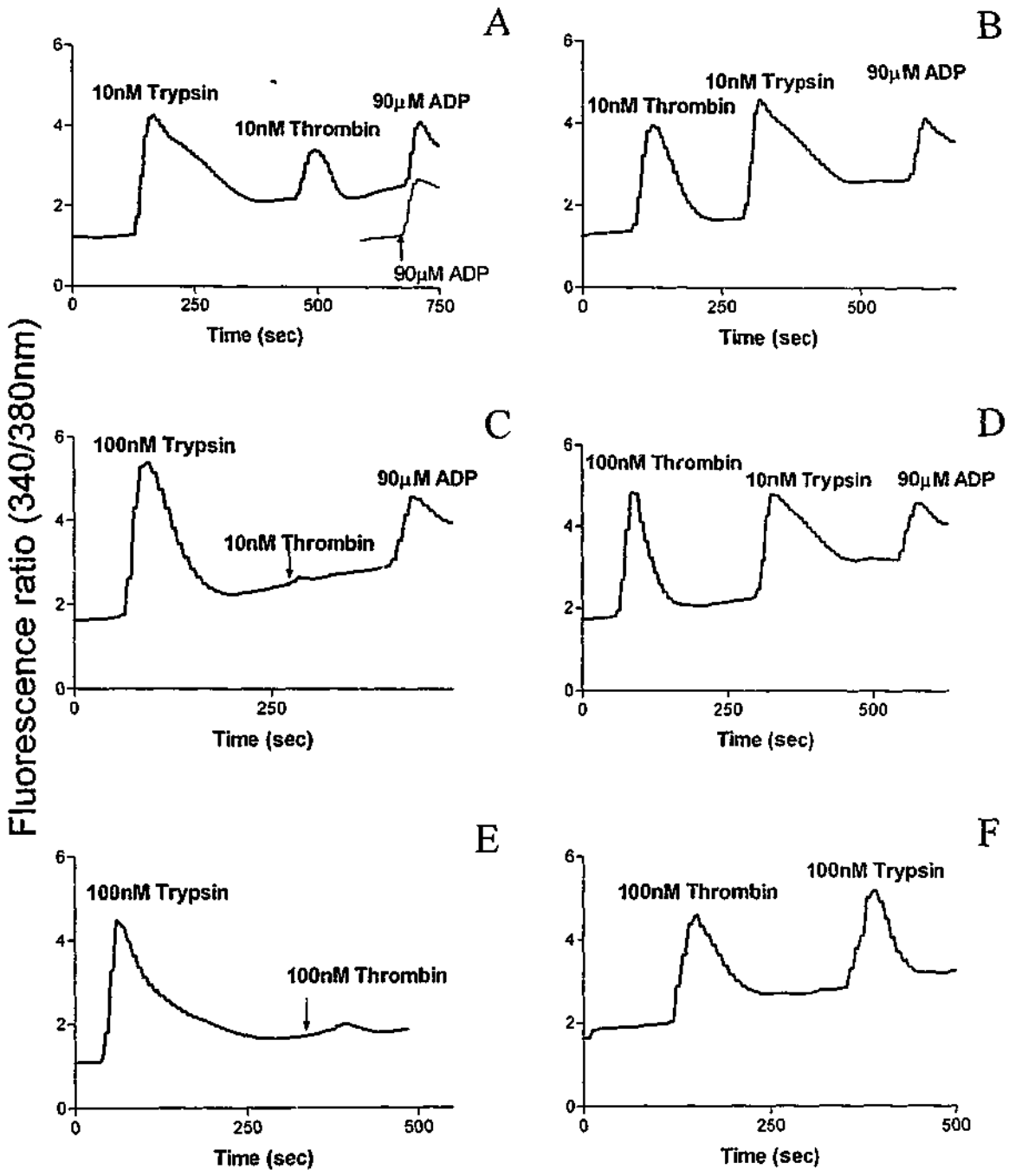


Figure 4.4. **Effects of desensitization of $[Ca^{2+}]_i$ mobilisation in response to trypsin and thrombin in KB60 cells.**

The $[Ca^{2+}]_i$ responses in KB60 cells to: A) 10 nM trypsin, followed by 10 nM thrombin, then 90 μM ADP (—) or 90 μM ADP alone (—); B) 10 nM thrombin, followed by 10 nM trypsin, then 90 μM ADP; C) 100 nM trypsin, followed by 10 nM thrombin then 90 μM ADP; D) 100 nM thrombin, followed by 10 nM trypsin then 90 μM ADP; E) 100 nM trypsin followed by 100 nM thrombin; F) 100 nM thrombin, followed by 100 nM trypsin.

Similarly, treatment of KB-60 cells with 20 nM RgpB reduced a subsequent response to 100 nM thrombin by approximately 90% (Fig 4.5A), whereas exposure of KB-60 cells to 100 nM thrombin reduced a secondary response to 20 nM RgpB by approximately 10% (Fig 4.5B). Treatment of KB-60 cells with 10 nM RgpB reduced a subsequent response to 10 nM thrombin by 60% whereas treatment of cells with 10 nM thrombin reduced a secondary response to 10 nM RgpB by 20%; in each instance the cells could efficiently respond to ADP (data not shown). It was shown that the desensitisation of the responses was not due to RgpB inactivating thrombin. This is illustrated by the use of antipain at a concentration known to inhibit RgpB effectively without affecting thrombin (Fig. 4.5C). When added to the cell suspension after the initial activation with RgpB, the antipain did not affect the desensitisation to a subsequent thrombin response (Fig. 4.5C & D). RgpB therefore exhibited a similar ability to trypsin in efficiently desensitizing subsequent thrombin responses.

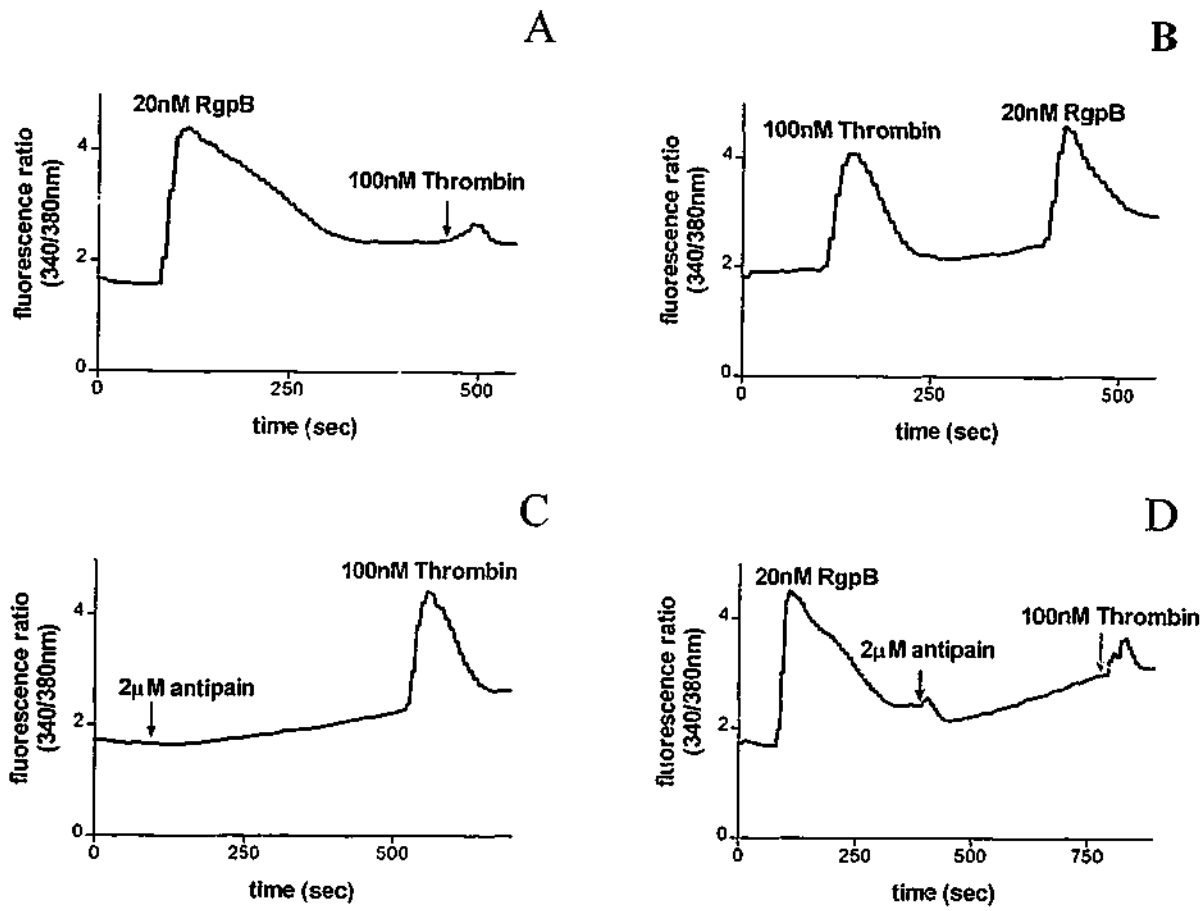


Figure 4.5. **Effects of desensitization of $[Ca^{2+}]_i$ mobilisation in response to RgpB and thrombin in KB60 cells.**

Cells were exposed to: A) 20 nM RgpB, followed by 100 nM thrombin; B) 100 nM thrombin, then 20 nM RgpB; C) 2 μM antipain, followed by 100 nM thrombin; D) 20 nM RgpB, followed by 2 μM antipain and then 100 nM thrombin.

When KB-60 cells were initially activated with 100 nM trypsin, a subsequent response to 100 nM trypsin was abolished (Fig 4.6A). Similarly, following treatment with 20 nM RgpB, a secondary response to this enzyme was eliminated (Fig 4.6B). Exposure of KB-60 cells to 10 nM trypsin virtually eliminated the $[Ca^{2+}]_i$ response to a second challenge with 10 nM RgpB (Fig 4.6C). Similarly, treatment of KB-60 cells with 10 nM RgpB desensitised the response to a second challenge by 10 nM trypsin (Fig. 4.6D), whereas treatment of KB-60 cells with trypsin or RgpB did not reduce the response to a subsequent challenge with ADP (Fig. 4.6C &D) or bradykinin (data not shown). Addition of 100 nM trypsin decreased a subsequent challenge with 20 nM RgpB by 90% (Fig. 4.6E) and similarly treatment with 20 nM RgpB abolished a secondary response to 100 nM trypsin (Fig. 4.6F). Therefore RgpB effectively desensitises the calcium mobilisation induced by trypsin and *vice versa*.

These desensitisation studies suggest that RgpB and trypsin activate common receptor(s) on KB-60 cells. Trypsin can activate PAR-1 and PAR-2 on the surface of KB-60 cells by cleaving after an arginine residue in their extracellular domain. Since RgpB has high specificity of cleavage, limited to peptide bonds after arginine residues, and has also been shown to activate human PAR-2 (Chapter 3) on cells stably expressing this receptor, the calcium response elicited in KB60 cells is probably due to activation of PAR-1 and PAR-2 by this bacterial enzyme.

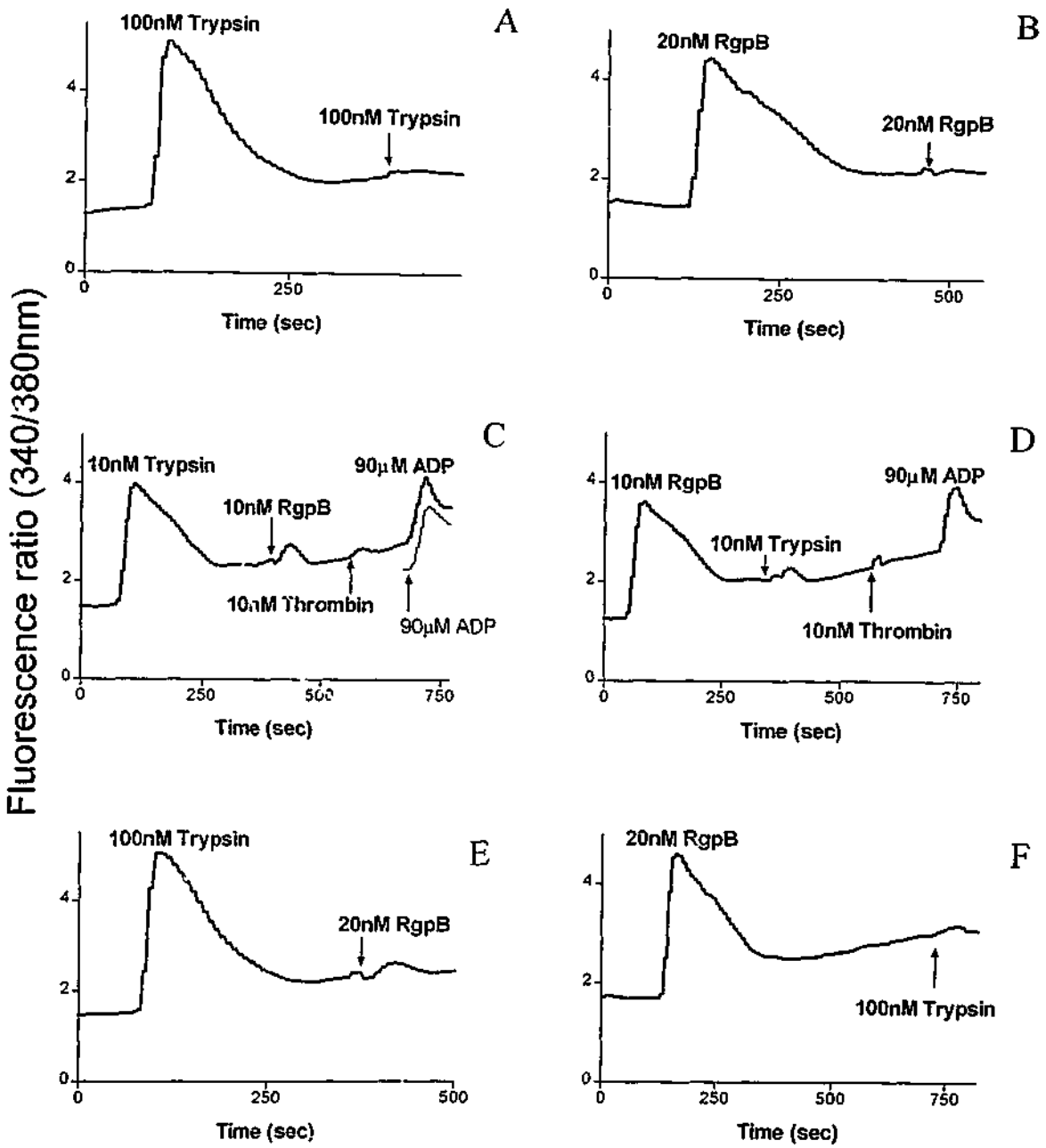


Figure 4.6. **Effects of desensitization of $[Ca^{2+}]_i$ mobilisation in response to trypsin and RgpB in KB60 cells**

Cells were treated with: A) 100 nM trypsin, then 100 nM trypsin; B) 20 nM RgpB, followed by 20 nM RgpB; C) 10 nM trypsin, followed by 10 nM RgpB, then 10 nM thrombin and finally 90 µM ADP (—) or 90 µM ADP alone (←); D) 10 nM RgpB, followed by 10 nM trypsin, then 10 nM thrombin and finally 90 µM ADP; E) 100 nM trypsin, then 20 nM RgpB; F) 20 nM RgpB, followed by 100 nM trypsin.

4.1.3 RgpB activates cells stably expressing human PAR-1

To demonstrate that RgpB can cleave and activate human PAR-1, the calcium response elicited by this enzyme was investigated in N1LF-PAR-1 cells in comparison to non-transfected N1LF cells. Treatment of N1LF-PAR-1 cells with 10 nM thrombin (Fig. 4.7B), elicited an intracellular Ca^{2+} increase, while non-transfected N1LF cells did not respond to 10 nM thrombin (Fig. 4.7A) nor did they respond to concentrations of thrombin as high as 100 nM (data not shown). Similarly, trypsin elicited calcium mobilisation in N1LF-PAR-1 cells (Fig. 4.7F) but not in control non-transfected N1LF cells (Fig. 4.7E). RgpB at a concentration of 2 nM elicited a $[Ca^{2+}]_i$ increase in N1LF-PAR-1 cells, whereas concentrations of the enzyme as high as 100nM did not induce a calcium response in non-transfected N1LF cells (data not shown). Treatment with 18nM RgpB caused an intracellular $[Ca^{2+}]_i$ increase in N1LF PAR-1 cells (Fig. 4.7C). In contrast, non-transfected N1LF cells did not respond to 18 nM RgpB (Fig. 4.7D), but did respond to bradykinin, to demonstrate that the cells were still capable of mobilising intracellular calcium. Thus, RgpB activates PAR-1.

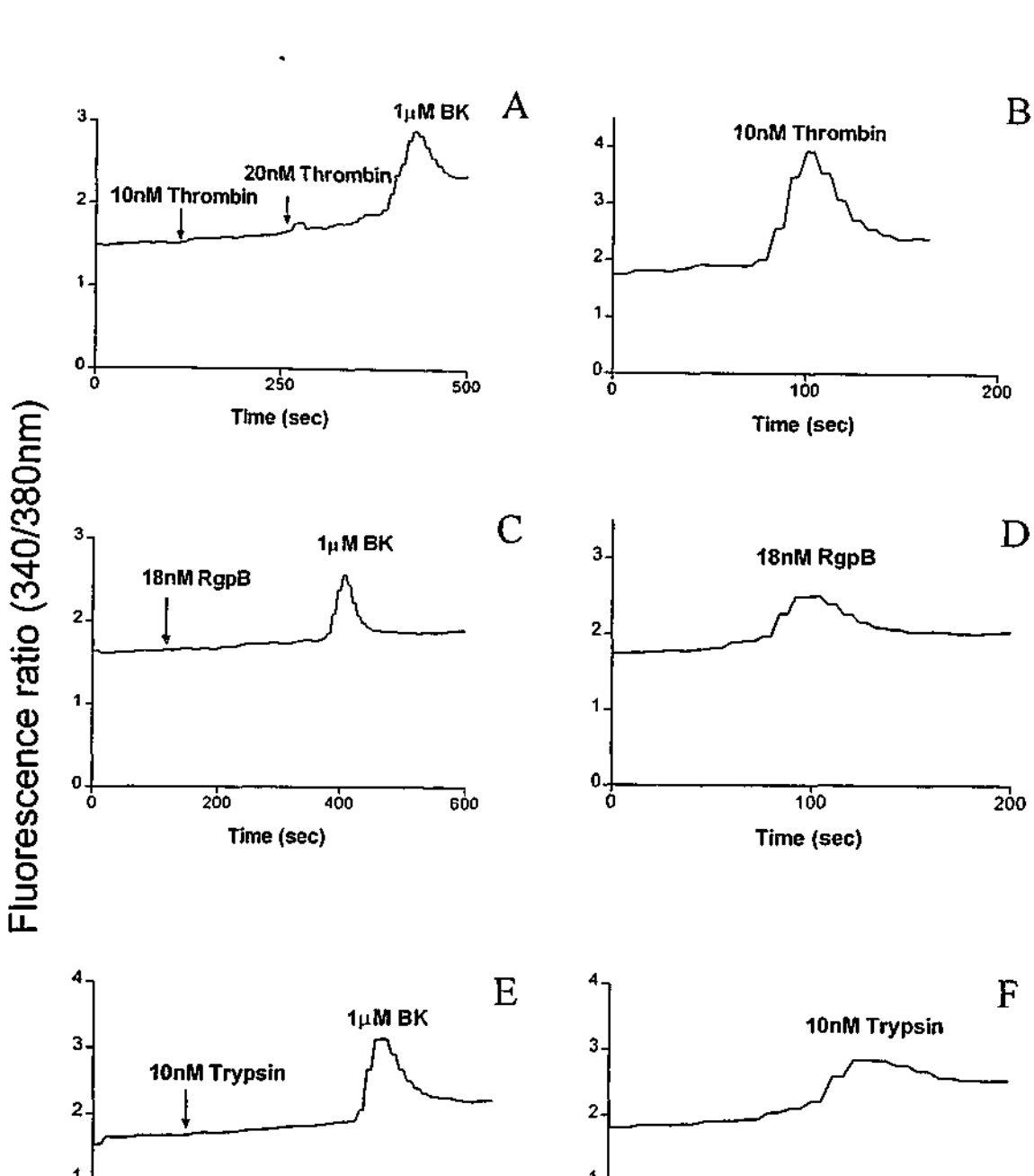


Figure 4.7. **Thrombin, RgpB and Trypsin activate PAR-1 on transfected cells stably expressing human PAR-1.**

Calcium mobilization in N1LF cells (A, C, E) or N1LF PAR-1 cells (B, D, F) induced by (A, B) 10 nM thrombin; (C, D) 18 nM RgpB and (E, F) 10 nM Trypsin. N1LF cells responded to 1 μM bradykinin in each case. The traces are representative of three similar sets of data obtained.

4.1.4 Treatment of KB-60 cells with RgpB causes an increase in secretion of IL-6

Human keratinocytes have been found to express PAR-1 and PAR-2 and their activation with their respective agonist peptides or trypsin and thrombin was found to induce IL-6 up-regulation (Wakita *et al.*, 1997). We investigated whether treatment with RAP, TRAP, trypsin, thrombin or RgpB could increase IL-6 secretion in KB-60 cells.

KB-60 cells were stimulated with RAP or TRAP (100 μM) for up to 1 hr and both peptides were found to cause an increase in IL-6 secretion. Preliminary studies showed that maximal concentrations of IL-6 were produced in the supernatant after treatment with RAP or TRAP for 1 hr and this time point was used for further analysis. It was found that TRAP (100 μM) stimulated IL-6 production at levels that were higher than those induced by RAP (100 μM) (Fig. 4.8A). IL-6 levels in supernatant from cells treated with RAP were found to be 2.5-fold higher than those measured in supernatants from control (non-treated) KB60 cells, whereas in supernatant from cells treated with TRAP, IL-6 secretion was 4-fold higher than that from untreated cells. RAP activates PAR-2, whereas TRAP has been found to activate both PAR-1 and PAR-2 and thus it may be that by acting through both these receptors, TRAP elicits a higher production of IL-6 compared to RAP.

KB-60 cells were treated with various concentrations of trypsin or thrombin for up to 1hr and both proteases were found to induce IL-6 up-regulation. Treatment of KB60 cells with trypsin or thrombin that had previously been inactivated resulted in levels of IL-6 secretion that were identical to the untreated cells, indicating that the cytokine increase observed was dependent on the proteolytic activity of the enzymes. Treatment of cells for 1 hr with trypsin (2.5 nM) was found to cause a higher production of IL-6 than thrombin (2.5 nM) (Fig. 4.8B). Thrombin and trypsin were found to cause a

2.5- and 3.5-fold increase in IL-6 secretion respectively, compared to untreated cells (Fig. 4.8B).

Treatment of KB60 cells with RgpB (2.5 nM) (previously treated with polymyxin B-SO_4 to inhibit any stimulation by bacterial lipopolysaccharides) for 1 hr induced a 3-fold increase in IL-6 secretion compared to untreated cells (Fig. 4.8C). RgpB that had been inactivated by antipain did not cause an increase in IL-6 production (Fig. 4.8C), indicating that the IL-6 up-regulation is due to the proteolytic activity of RgpB. Treatment of the cells for 15 min indicated that the increase in IL-6 secretion caused by RgpB was dependent on the concentration of the protease used (Fig 4.8D). TNFα and IFN-γ levels were measured in supernatants from KB60 cells following treatment with trypsin, thrombin or RgpB (2.5 nM) for up to 1 hr and were found to be the same as those from untreated control cells (data not shown).

Following treatment of KB60 cells with thrombin, trypsin or RgpB (2.5 nM) for 1 hr, 95% of cells were viable as assessed by trypan blue exclusion. No changes in cell morphology or cell detachment were observed during treatment with thrombin, trypsin or RgpB (2.5 nM) for 1 hr. The appearance of cells treated with RgpB for 1 hr is seen in Fig. 4.9. KB60 cell detachment was only observed following treatment for more than 2 hrs with 2.5 nM RgpB or following treatment for 30 min with 25 nM RgpB.

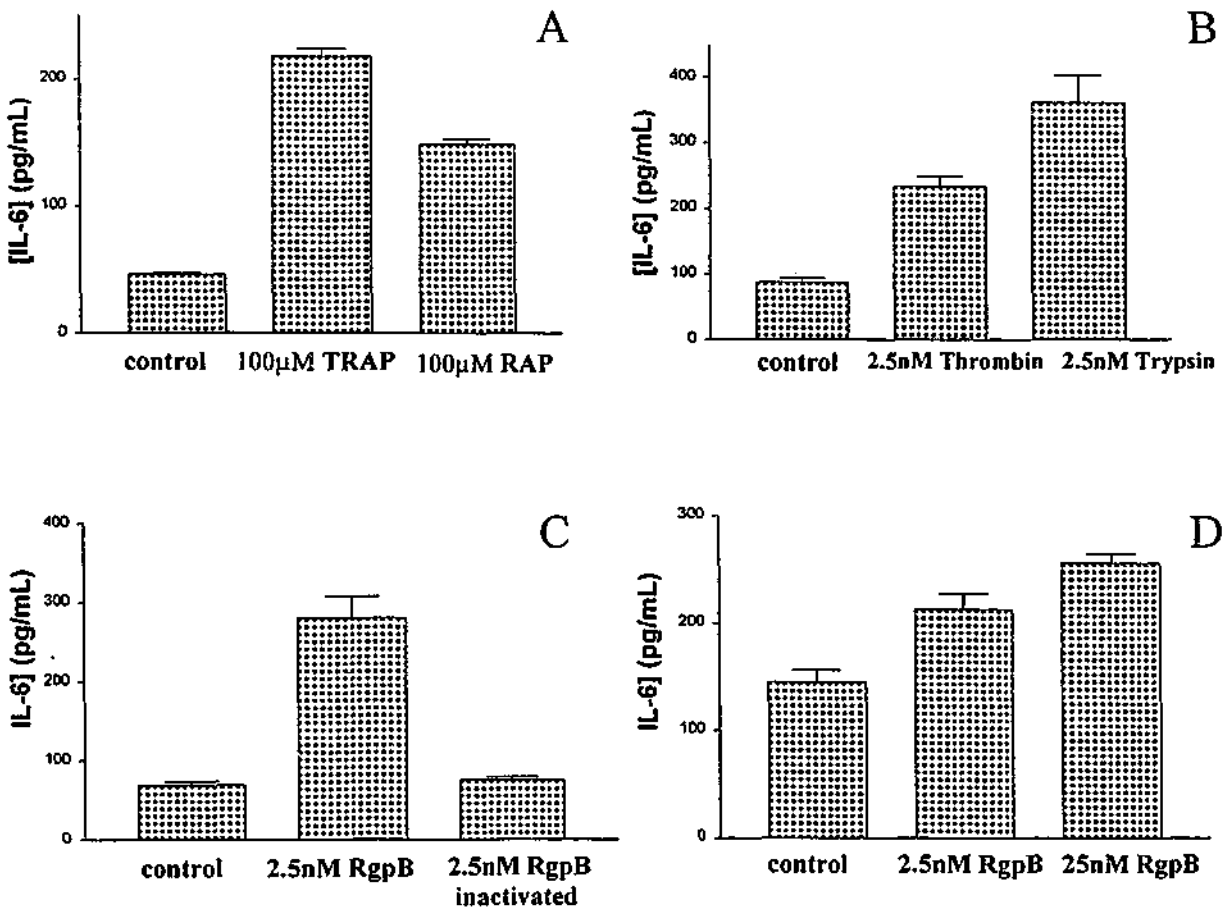
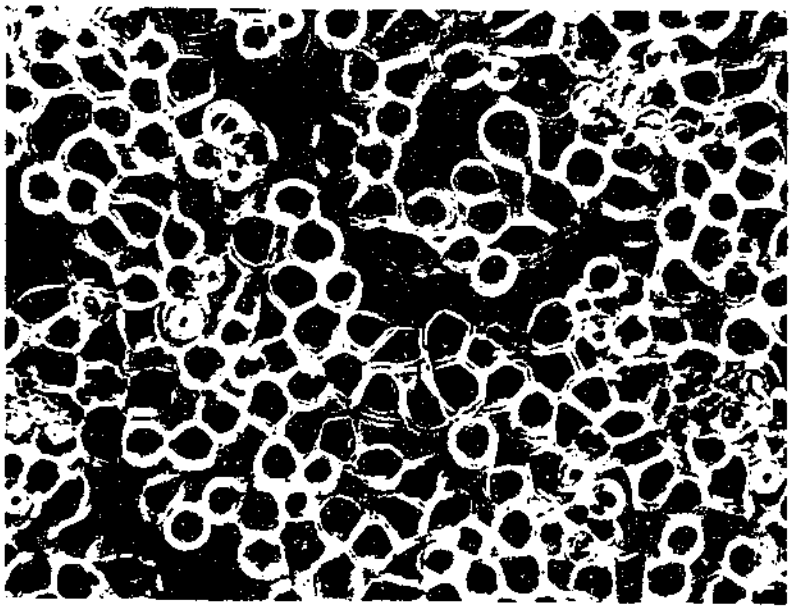
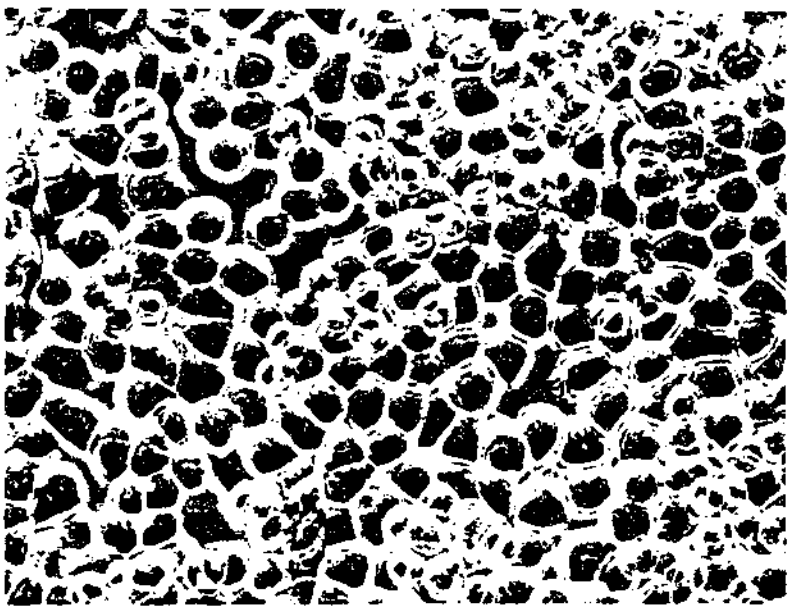


Figure 4.8. **Thrombin, trypsin, RgpB, TRAP and RAP-induced up-regulation in IL-6 secretion in KB-60 cells**

Measurement of IL-6 concentrations in culture supernatants from KB60 cells by ELISA following treatment for 1hr (A-C) or 15 min (D) with the indicated agonists: A) 100 μM TRAP or 100 μM RAP; B) 2.5 nM thrombin or 2.5 nM trypsin; C) 2.5 nM RgpB or 2.5 nM RgpB inactivated with antipain; D) 2.5 nM RgpB or 25 nM RgpB. The results shown in A, B and D represent the mean ± SEM obtained from three experiments and those shown in C are results obtained from five experiments ***P<0.001.



A



B

Figure 4.9. **Cell shape and morphology of KB60 cells following treatment with RgpB.**

Phase contrast microscopy of KB60 cells following treatment for 1hr with A) 10 mM cysteine buffer; B) 2.5 nM RgpB.

Molecules known to be important in cell to cell or cell to matrix adhesion of epithelial cells include E-cadherin, occludin and β_1-integrin. KB60 cells do not express occludin and E-cadherin (Katz *et al.*, 2000). To investigate whether RgpB up-regulates IL-6 production by affecting cell to cell or cell to matrix adhesion molecules, the cell-surface expression of β_1-integrin was examined by indirect immunofluorescence and cell-ELISA assay. Following treatment with 2.5 nM RgpB (activated in cysteine buffer) for 1 hr it was found that the pattern of distribution of β_1-integrin was the same as cells treated with cysteine buffer alone (Fig. 4.10). The pattern of β_1-integrin distribution is consistent with cell surface expression and the staining appears to be more intense in areas of cell-cell contact. The level of expression of β_1-integrin on cells treated with RgpB was determined using a cell ELISA assay. Cells were plated in a 96 well plate at two different densities to verify that the cell-ELISA assay was sensitive for detection of different concentration of cells. Following treatment with 2.5 nM RgpB (activated in cysteine buffer) for 1 hr (n=4) it was found that the level of expression of β_1-integrin was the same as cells treated with cysteine buffer alone (n=4) (Table 4.1).

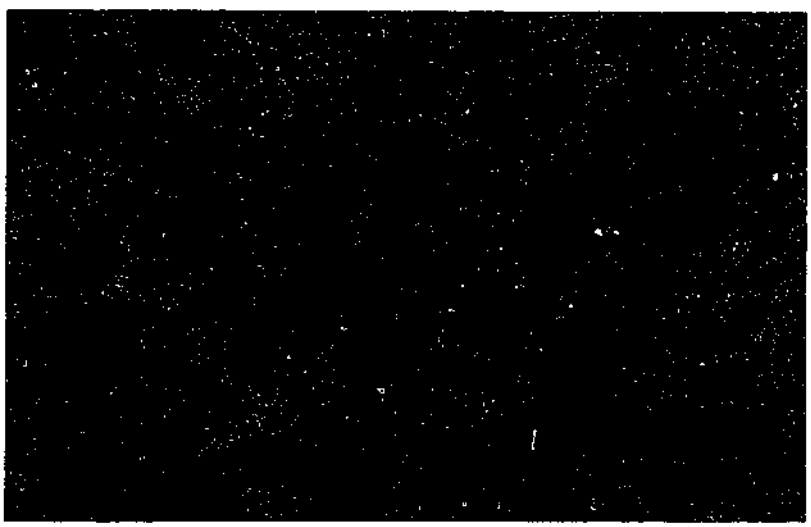
Table 4.1 **Expression of integrin-β_1 on KB60 cells treated with RgpB.**

Cell-ELISA assay of KB60 cells following treatment for 1 hr with 2.5 nM RgpB or 10 mM cysteine buffer. The results represent the mean ± SEM.

A_{450nm}

10×10^4 cells/mL		4×10^4 cells/mL	
Control buffer	**RgpB (2.5 nM)**	**Control buffer**	**RgpB (2.5 nM)**
1.901± 0.119	1.768 ± 0.039	0.664 ± 0.007	0.689 ± 0.012

A



B

Figure 4.10. **Distribution of integrin-β_1 on KB60 cells treated with RgpB.**
Fluorescence microscopy of KB60 cells stained with integrin-β_1 antibody following treatment for 1hr with A) 10 mM cysteine buffer; B) 2.5 nM RgpB. Exposure time was 50sec for each fluorescence micrograph.

4.2 Discussion

Chronic marginal gingivitis is characterised by gingival inflammation, redness, oedoma, bleeding, changes in contour of the gingiva and increased flow of gingival crevicular fluid. Hallmarks of periodontitis, which is a progression of gingivitis, are the loss of gingival connective tissue attachment to the teeth, gum regression, loss of periodontal ligament, which attaches the tooth to the surrounding alveolar bone, tooth loss and alveolar bone resorption. Refractory periodontitis is characterised by its relative resistance to repeated therapeutic procedures aimed at controlling the progression of periodontal tissue destruction (Schenkein, 1999).

The gingival epithelium is directly exposed to periodontal bacteria and their products and, by receiving and transmitting signals, plays an important role in the overall dialogue that occurs between pathogens and the host. In this study we showed that protease-activated receptors-1, -2 and -3, which elicit a number of cellular responses, are expressed on the surface of human epidermoid KB60 cells. PAR-1 and PAR-2 agonist peptides along with trypsin, a known activator of PAR-1 and PAR-2, and thrombin, which activates PAR-1 and PAR-3, induced calcium mobilization in these cells. We then investigated whether an arginine-specific protease from the bacterium *P. gingivalis*, RgpB, could interact with these receptors. RgpB induced an increase in $[Ca^{2+}]_i$ in human KB60 cells which was dependent upon its proteolytic activity. Several findings strongly support the hypothesis that this increase is mediated by activation of PAR-1 and PAR-2. Treatment of KB60 cells with RgpB desensitized the $[Ca^{2+}]_i$ response to a second challenge with the same enzyme, a phenomenon that is in agreement with the rapid desensitization that protease-activated receptors undergo after a short period of activation with a protease. Desensitization studies carried out with trypsin showed that when KB60 cells were activated with this enzyme, a secondary

challenge with RgpB was eliminated. Similarly, exposure of KB60 cells to the bacterial enzyme desensitized the response to a second challenge with trypsin, suggesting RgpB is activating the same receptors on KB60 cells as trypsin, namely PAR-1 and PAR-2.

Pre-treatment of KB60 cells with trypsin drastically reduced a subsequent challenge with thrombin, whereas treatment of the cells with thrombin reduced a subsequent response to trypsin by approximately 20%. Since PAR-1, PAR-2 and PAR-3 are expressed on the surface of KB60 cells, trypsin would be expected to desensitise PAR-1 and PAR-2, whereas PAR-3 would be available for cleavage by thrombin. The finding that the response to thrombin was minimal subsequent to trypsin treatment indicates that PAR-1 mediates the majority of the calcium mobilization induced by thrombin and PAR-3 represents a minimal portion, if any, of the thrombin response. Similarly, O' Brien *et al.*, (2000) detected PAR-1, PAR-2 and PAR-3 mRNA but not PAR-4 in human umbilical vein endothelial cells (HUVEC) and found that thrombin-induced inositol accumulation in HUVEC was reduced by 93% in the presence of PAR-1 blocking antibodies, making a contribution from PAR-3 appear minimal if not unlikely. O'Brien *et al.*, (2000) postulated that PAR-3, which has been found to be expressed on the surface of HUVEC cells (Schmidt *et al.*, 1998), may actually be unable to mediate a thrombin response on its own in the absence of PAR-4, which appears to be the case in mouse platelets (Coughlin *et al.*, 2000). Treatment with thrombin would cause desensitisation of PAR-1 and PAR-3, leaving PAR-2 intact and available for activation by a secondary treatment with trypsin. The fact that thrombin reduced a secondary response to trypsin only to a small degree allows the suggestion that the majority of the trypsin-induced calcium mobilisation is induced by activation of PAR-2 and only a small percentage of the trypsin response is due to PAR-1 activation. Similar to trypsin, RgpB efficiently desensitised subsequent responses to thrombin whereas thrombin only

marginally reduced a secondary response to the bacterial enzyme, suggesting that RgpB acts mainly through PAR-2 and to a lesser extent through PAR-1 to cause calcium mobilisation.

Both PAR-1 and PAR-2, expressed on the surface of KB60 cells, are activated following cleavage after a specific arginine residue in their extracellular domain. RgpB, which is absolutely specific for hydrolysis at Arg-Xaa sites, was found to activate both PAR-1 and PAR-2 (Chapter 3) in transfected cells stably expressing these receptors. Based on the desensitization studies and the ability of RgpB to activate PAR-1 and PAR-2 on transfected cells, it can be concluded that the bacterial protease activates these receptors on the surface of KB60 cells, resulting in calcium mobilization. It should be noted that, due to the specificity of these proteinases for cleavage after arginine residues, they are unable to activate PAR-3, which has a lysine residue at the cleavage point required to activate the receptor.

Activation of PAR-1 and PAR-2 on KB60 cells by their respective agonist peptides was found to cause an increase in IL-6 secretion. Similarly treatment of KB60 cells with either thrombin or trypsin resulted in IL-6 up-regulation, which was due to the proteolytic activity of the enzymes. Treatment of KB60 cells with TRAP, which activates both PAR-1 and PAR-2, caused a higher increase in IL-6 levels compared to RAP, which activates only PAR-2. Similarly, trypsin, which cleaves both PAR-1 and PAR-2, resulted in a greater stimulation of IL-6 expression compared to thrombin, which appears to activate mainly PAR-1 based on the calcium desensitization studies. Exposure of KB60 cells to RgpB also resulted in an increase in IL-6 secretion, which was found to be dependent upon the proteolytic activity of the enzyme. During the period of treatment no changes in cell shape or attachment were observed.

It has recently been reported that *P. gingivalis* cells can destroy epithelial cell-cell junction complexes in canine kidney cells by degrading β_1-integrin, occludin and

E-cadherin, possibly through the action of proteases produced by the bacterium (Katz *et al.*, 2000). Following incubation of canine kidney cells with *P. gingivalis* cells for 2 hrs it was found that there is "a critical threshold concentration of *P. gingivalis* cells (>10^9 cells/mL) that is required in order to cause epithelial barrier destruction" (Katz *et al.*, 2000). Scragg *et al.* (1999) have shown that gingipains-R of *P. gingivalis* can degrade $\alpha_5\beta_1$-integrin on human gingival fibroblasts, and it was observed that a threshold concentration of proteases was necessary for integrin cleavage over the time period tested. KB60 cells do not express occludin and E-cadherin (Katz *et al.*, 2000). In the current study, under the conditions that increased IL-6 secretion, no changes in pattern of distribution or expression levels of integrin-β_1 were observed. This indicates that the increase in IL-6 secretion induced by RgpB is not mediated by degradation of integrin-β_1. The up-regulation of IL-6 secretion from KB60 cells following treatment with RgpB is of particular interest as IL-6 is a pro-inflammatory cytokine associated with periodontal disease.

Cytokines are molecules released by cells in their local environment and have chemoattractant properties for inflammatory cells. Cytokines that are readily found in periodontium and gingival crevicular fluid of patients with periodontal disease are IL-6, IL-8, IL-1, TNF-α and PGE_2 (Schenkein, 1999; Beck *et al.*, 1996). It has been suggested that cytokines play significant roles in the pathogenesis of periodontitis (Kjeldsen *et al.*, 1993; Wilson *et al.*, 1996). In the majority of investigations carried out, IL-6 expression was found to be higher at sites of periodontal inflammation (Irwin and Myrillas, 1998). IL-6 levels were found to be increased in diseased gingiva from patients with periodontitis compared to gingiva from periodontally healthy subjects (Takahashi *et al.*, 1994; Chen *et al.*, 1997). Compared to normal tissue, IL-6 expression appears to be elevated in inflamed tissue from periodontitis sites and is highest in gingivitis sites (Matsuki *et al.*, 1992; Yamazaki *et al.*, 1994). Studies have indicated

that IL-6 levels are higher in gingival crevicular fluid from refractory periodontitis patients and may also correlate with progression of disease in these patients (Reinhardt *et al.*, 1993; Lee *et al.*, 1995; Geivelis *et al.*, 1993). IL-6 stimulates plasma cell proliferation and therefore may promote the presence of plasma cells that are readily found in periodontitis lesions (Schenkein, 1999). IL-6 is secreted by human osteoblasts in response to bone resorbing agents (Littlewood *et al.*, 1991), promotes bone resorption (Ishimi *et al.*, 1990), acts as a potent inducer of osteoclast formation (Kurihara *et al.*, 1990; Mihara *et al.*, 1995) and inhibits bone formation *in vitro* (Hughes and Howells, 1993). Thus IL-6 is likely to contribute to the bone resorption associated with periodontal diseases (Schenkein, 1999).

Other studies reported degradation of cell surface molecules following incubation of cells with *P. gingivalis* and its products over extended periods of time (Katz *et al.*, 2000; Scragg *et al.*, 1999). In this study it was found that very low concentrations of RgpB were sufficient to immediately induce an increase in $[Ca^{2+}]_i$ in KB60 cells, indicating that RgpB is very efficient in eliciting an intracellular signal by activating the PARs. Interestingly, following contact with epithelial cells, invading *P. gingivalis* bacterial cells cause a transient $[Ca^{2+}]_i$ increase in these cells (Izutsu *et al.*, 1996), thus it may be of interest to determine whether PAR activation by the bacterium's proteases is involved in this process.

The findings presented here provide evidence that PAR-1, PAR-2 and PAR-3 are expressed on the surface of epidermoid cells and that PAR-1 and PAR-2 can be activated by the arginine-specific bacterial protease, RgpB. Cleavage of the PARs and the resultant up-regulation of IL-6 secretion by the bacterial protease are likely to contribute to the local inflammatory reaction within the pathological periodontal pocket and the propagation of the chronic inflammatory condition present in periodontal disease.

CHAPTER 5

RgpB and HRgpA from *Porphyromonas gingivalis* Activate PAR-1 and PAR-4 on Human Platelets and Induce Platelet Aggregation

5.0 Introduction

Atherosclerosis is a progressive disease affecting elastic arteries. In advanced stages a lesion called an atheroma is formed which consists of elevated focal intimal plaques containing low density lipoproteins, cholesterol and plasma proteins including fibrin and fibrinogen. This atheromatous plaque is infiltrated with proliferating arterial smooth muscle cells along with monocytes and macrophages. Lipid accumulates in these cells causing cell death and the formation of a necrotic core. Rupture of the surface of the atherosclerotic plaque allows contact of flowing blood with molecules that activate platelets, promoting platelet aggregation and thrombus formation, placing patients with atherosclerosis at high risk of thrombosis (Lowe, 1998; Lusis, 2000; Libby, 2000; Hegele, 1996). Clinical complications of atherosclerosis include coronary heart disease and myocardial infarction.

In patients with periodontitis the most commonly found associated condition is heart disease (Umino and Nagao, 1993; Nery *et al.,* 1987). In two studies (Mattila *et al.,* 1989a; Mattila *et al.,* 1993) using 100 patients with acute myocardial infarction and 102 controls, it was found that the heart disease patients had worse dental health than the controls. It was also reported that the relationship between poor dental health and heart disease was not dependent on age, total cholesterol, triglycerides, C peptide, hypertension,

presence of diabetes and smoking (Mattila *et al.*, 1989a). In another study (Paunio *et al.*, 1993) which examined 1,384 men aged 45-64 it was found that the number of missing teeth was related to the prevalence of ischemic heart disease and was independent of age, hypertension, geographic area, educational level, smoking and cholesterol. Beck *et al.* (1996) analysed the data of a cohort study by combining the data of a dental longitudinal study and a normative aging study. Mean tooth alveolar bone loss scores were measured in 1,147 men and their health was examined over a period of 18 years. It was found that 207 men developed coronary heart disease (CHD) and 59 died of CHD. Incidence odds ratios for the relationship between the periodontal disease measures and CHD were adjusted for age, educational level, as well as cardiovascular risk factors, ie. alcohol consumption, cholesterol, blood pressure, body mass index, smoking and family history of heart disease. Incidence odds ratios between bone loss and CHD or fatal CHD were found to be 1.4 and 1.6, respectively. (Incidence odds ratio of 1.4 means that for every 20% increase in mean bone loss, the incidence of total CHD increases 40%). Increasing levels of bone loss were found to be accompanied by a higher cumulative frequency of occurrence of CHD and fatal CHD, indicating a biological gradient between severity of exposure and occurrence of disease. Loesche (1994) presented findings that indicate that reported history of CHD is inversely proportional to the number of teeth. In another study the incidence of CHD and periodontal disease and number of teeth lost was examined in 44,119 men, over a 6 year period. The findings suggested a positive relationship between tooth loss and CHD, although the investigators pointed out that other factors such as diet and socioeconomic status should be explored (Joshipura *et al.*, 1998).

An extensive study has been presented by DeStefano *et al.*, (1993) who analysed data from a National Health and Nutrition Epidemiologic Follow-Up Study in the USA.

The dental health of 20,749 subjects aged between 25 to 74 years was examined. The health of 90% of the subjects was monitored over a period of 14 years and the incidence of mortality or admission to hospital due to coronary heart disease was measured. Subjects with periodontitis had a 25% increased risk of coronary heart disease relative to those with minimal periodontal disease. In men younger than 50 years old, there was a strong association between periodontal disease and coronary heart disease. Men with periodontitis or who had no teeth were 70% more likely to have coronary heart disease than men with no periodontal disease. Taking into account age, sex, race, education, poverty index, marital status, systolic blood pressure, total cholesterol, diabetes, body mass index, physical activity, alcohol consumption, and cigarette smoking, men with periodontitis had a 1.72 relative risk compared to men without periodontitis.

These studies indicate an association between periodontal infections and coronary heart disease. Infections with pathogens such as *Chlamydia pneumoniae* and *Helicobacter pylori* have been recognised as a risk factor for coronary heart disease and atherosclerosis (Saikku *et al.*, 1988; Mendall *et al.*, 1994; Patel *et al.*, 1995). Periodontal infections have been associated with atherosclerosis and thromboembolic events (Mattila *et al.*, 1993; Mattila *et al.*, 1989b). The atherosclerotic mass was estimated using an angiogram in patients with myocardial infraction compared to controls, and a significant association was found between dental infections and coronary atheromatosis (Mattila *et al.*, 1989a). Other studies have provided more information at a molecular level on the relationship between dental infections and atherosclerosis and thrombosis. As in many other infectious diseases, plasma fibrinogen levels and leukocyte counts are increased in individuals with periodontal disease (Lowe *et al.*, 1992; Kweider *et al.*, 1993) and high levels of factor VIII activity and

its cofactors in the coagulation pathway are associated with poor dental health (Mattila *et al.*, 1989c), linking chronic dental infection with increased thrombogenicity.

A hypothetical model postulates that lipopolysaccharides (LPS) may play a role in infection-associated atherogenesis, as LPS is produced by periodontal pathogens and is found in the gingival crevicular fluid (GCF) (Lopes-Virella and Virella, 1985; Beck *et al.*, 1996; Page, 1998). Indeed, significantly increased levels of pro-inflammatory cytokines such as IL-1β, TNF-α and IL-6, as well as other pro-inflammatory active compounds including PGE_2 and TxB_2 have been found in GCF (Eley & Cox, 1998; Beck *et al.*, 1996). Infusion of lipopolysaccharides (LPS) up-regulates the expression of endothelial adhesion molecules, triggers the release of IL-1β, TNF-α and TxB_2, initiates platelet aggregation and adhesion, and promotes the formation of lipid-laden foam cells and the deposition of cholesterol in the intima (Marcus and Hajjar, 1993). In addition, IL-1β has been shown to favour coagulation and thrombosis while inhibiting fibrinolysis (Clinton *et al.*, 1991). Diseased aortas contain large amounts of IL-1β and TNF-α (Pearce *et al.*, 1992). Thus the hypothesis is that the peridontium can serve as a reservoir of LPS and cytokines that can also appear systemically and induce events associated with atherosclerosis (Beck *et al.*, 1996; Page, 1998).

Periodontal pathogens may play a direct role in the pathogenesis of atherosclerosis and thromboembolic events. The major pathogens in gum disease are the Gram-negative anaerobic bacteria *Porphyromonas gingivalis, Bacteroides forsythus and Actinobacillus actinomycetemcomitans*. Gram-negative periodontal pathogens do find their way into the blood stream (bacteraemia) in patients with periodontal disease as a result of oral hygiene procedures or even chewing (Silver *et al.*, 1980; Carroll and Sebor, 1980; Sconyers *et al.* 1973; Murray and Moosnick, 1941). An example that illustrates a connection between a

periodontal pathogen and thromboembolic events, is the effect of *Streptococcus sanguis* on platelets. *Streptococcus sanguis*, a benign gingival microbe, has also been identified in microbial bacteraemias from dental foci (Watanakunakorn and Pantelakakis, 1993). Certain strains of *S. sanguis* induce platelet aggregation *in vitro* (Herzberg *et al.*, 1983) as these strains express a protein named platelet aggregation-associated protein (PAAP) on their surface (Herzberg *et al.*, 1990). Such *S. sangius* strains appear to interact with circulating platelets, as infusion of *S. sangius* cells into rabbits caused formation of thromboemboli and cardiovascular and pulmonary changes that are consistent with the occurrence of myocardial infarction (Herzberg and Weyer, 1998). *S. sanguis* strains not expressing the PAAP protein did not cause any changes, indicating that these abnormalities are caused by the bacterial cells interacting with platelets (Herzberg and Weyer, 1998).

The effect of *P. gingivalis* on platelets is of interest, in light of the fact that bacteria found on the epidermis and the periodontium do find their way into the blood stream and that *P. gingivalis* has been found to infect atherosclerotic plaques (Haraszthy *et al.*, 1998; Chiu, 2000). This periodontal pathogen produces arginine-specific proteases named gingipains-R (Pike *et al.* 1994, Potempa *et al.* 1998) that are present as three secreted variants: RgpA, RgpB and HRgpA. HRgpA is the high molecular mass form of RgpA, formed by RgpA noncovalently complexed with haemagglutinins/adhesins (Rangarajan *et al.*, 1997). Curtis *et al.* (1993a) showed that a protease termed Protease I from *P. gingivalis* strain W83, with trypsin-like activity and the ability to cleave after arginine, was able to induce platelet activation, but the biochemical nature of the enzyme and the mechanism of its action was not elucidated (Curtis *et al.*, 1993a). The questions addressed in this study were whether the highly characterised proteases RgpB and HRgpA which are solely responsible for the hydrolysis of peptide bonds after arginine residues in

P. gingivalis, can elicit a cellular response in platelets that is mediated by interaction of the enzymes with platelet surface molecules.

Platelets express on their surface members of the protease-activated receptor (PAR) family (Kahn *et al.*, 1999). To date, four protease-activated receptors have been identified: PAR-1, PAR-2, PAR-3 and PAR-4 (Coughlin, 2000). Synthetic peptides (agonists) corresponding to the tethered ligand of each receptor are able to activate the receptor, with the exception of PAR-3. Thrombin activates both PAR-1 (Vu *et al.*, 1991) and PAR-4 (Kahn *et al.*, 1998a; Xu *et al.*, 1998) by cleaving their extracellular domain after an arginine residue. The cellular responses that PAR-1 and PAR-4 mediate can be distinguished and studied by treatment of cells with their respective agonist peptides. Human platelets express PAR-1 (Vu *et al.*, 1991, Hung *et al.*, 1992), and its agonist peptide (TRAP) causes platelet activation (Vu *et al.*, 1991, Vassallo *et al.*, 1992, Scarborough *et al.*, 1992). PAR-4 is also expressed in human platelets (Kahn *et al.*, 1999; Xu *et al.*, 1998), although at a lower level than that of PAR-1 (Kahn *et al.*, 1999; Xu *et al.*, 1998), and PAR-4 agonist peptide (TRAP-4) was also found to induce aggregation of human platelets (Kahn *et al.*, 1999). The possible interactions of RgpB and HRgpA with the protease-activated receptors expressed on the surface of platelets were investigated in this study and it was conclusively demonstrated that the enzymes are potent agonists for both PAR-1 and PAR-4, mediating platelet activation and aggregation via these receptors. This constitutes the first report of bacterial proteases demonstrably acting through these receptors to cause cellular processes, which may underlie reported associations between periodontal disease and cardiovascular disease.

5.1 Results

5.1.1 HRgpA and RgpB increased intracellular calcium levels $[Ca^{2+}]_i$ in platelets

In order to determine whether the gingipains were able to interact with platelet receptors and induce intracellular signals, platelets from 9 donors were isolated and individually tested for a $[Ca^{2+}]_i$ response to HRgpA, RgpB and thrombin. As may be seen in Table 5.1, most donors responded similarly to HRgpA and RgpB in terms of the magnitude of the response to a defined concentration of the bacterial enzymes. HRgpA consistently induced a higher level of $[Ca^{2+}]_i$ response than the same concentration of thrombin, while 10-fold more RgpB consistently gave responses which were considerably lower than thrombin. HRgpA and RgpB induced a dose-dependent increase in $[Ca^{2+}]_i$, indicated by the dose responses shown for two donors in comparison to thrombin (Fig. 5.1) These data yield an enzyme concentration inducing the half maximal response (EC_{50}) of 2.4 nM and 0.18 nM for thrombin and HRgpA respectively in one donor and 0.6 nM and 63 nM for HRgpA and RgpB respectively in a second donor. This further indicates that HRgpA is consistently a more potent platelet agonist than thrombin, while RgpB is much less potent than the other two enzymes. As indicated by the data in Table 5.1, the magnitude of the $[Ca^{2+}]_i$ response for thrombin, HRgpA and RgpB varied to only a moderate extent between donors. HRgpA and RgpB which had been inactivated by leupeptin did not induce calcium responses in the platelets (Fig. 5.2A & D), indicating that the $[Ca^{2+}]_i$ increase induced is due to the proteolytic activity of the enzymes. The magnitude of the HRgpA-induced increase in $[Ca^{2+}]_i$ in platelets was not affected by the absence of extracellular Ca^{2+} (data not shown).

Thus, the response to the gingipains reflected Ca^{2+} mobilization from intracellular stores and did not require Ca^{2+} entry from the extracellular space.

Table 5.1. **Intracellular calcium responses in human platelets from a range of donors to PAR agonists.**

	Fluorescence ratio (340/380nm)				
Donor	**TRAP (150 μM)**	**TRAP-4 (1mM)**	**Thrombin (1 nM)**	**HRgpA (1 nM)**	**RgpB (10 nM)**
1	4.52	1.45	2.23	4.33	1.24
2	2.17	1.55	2.4	3.61	1.02
3	3.92	1.46	2.2	3.45	0.92
4	3.02	1.47	1.93	3.72	0.77
5	2.41	1.55	2.57	4.15	1.1
6	1.81	nd	1.92	5.0	nd
7	nd	nd	2.24	3.35	0.94
8	nd	nd	0.6	2.2	0.39
9	nd	nd	2.27	3.93	1.21

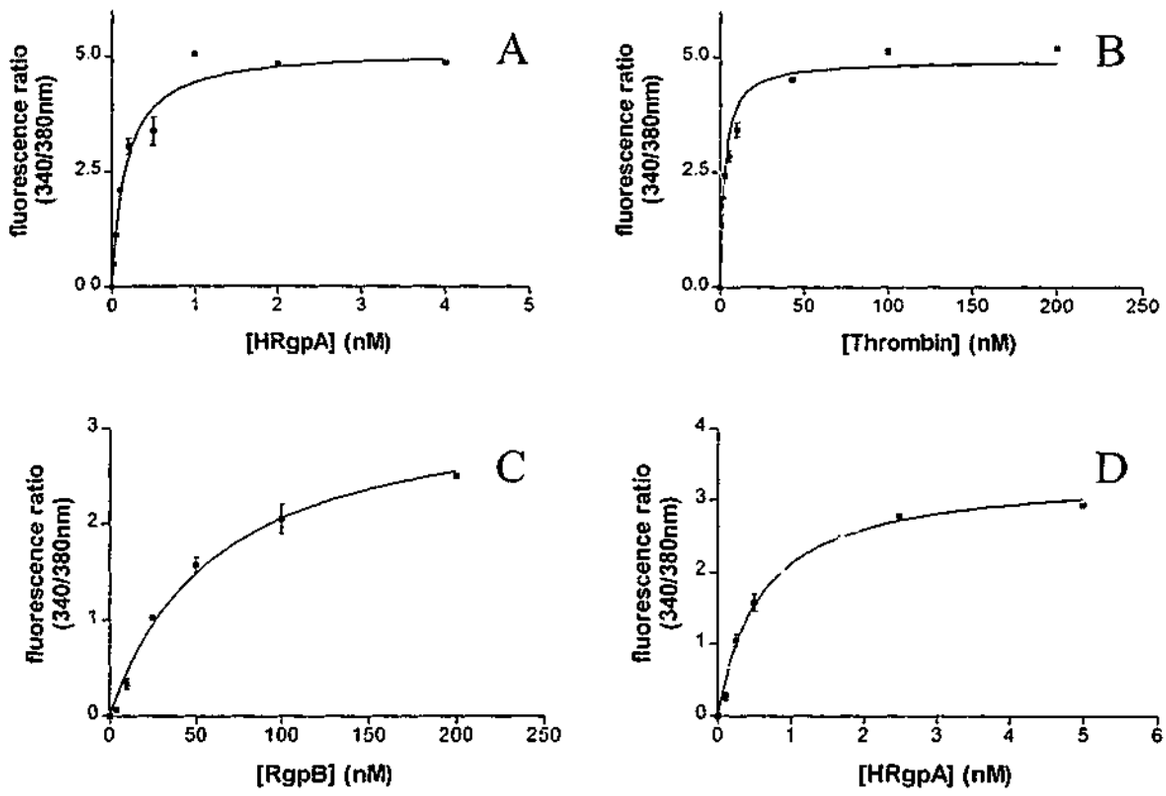


Figure 5.1. **The $[Ca^{2+}]_i$ response of platelets to different concentrations of HRgpA, RgpB and thrombin.**

Platelets from two donors were loaded with Fura-2 and stimulated with HRgpA, RgpB and thrombin. The responses in one donor to HRgpA (A) and thrombin (B) and in another donor to RgpB (C) and HRgpA (D) are shown. The $[Ca^{2+}]_i$ increases at different concentrations represent the mean from two traces similar to those shown in Fig. 5.2.

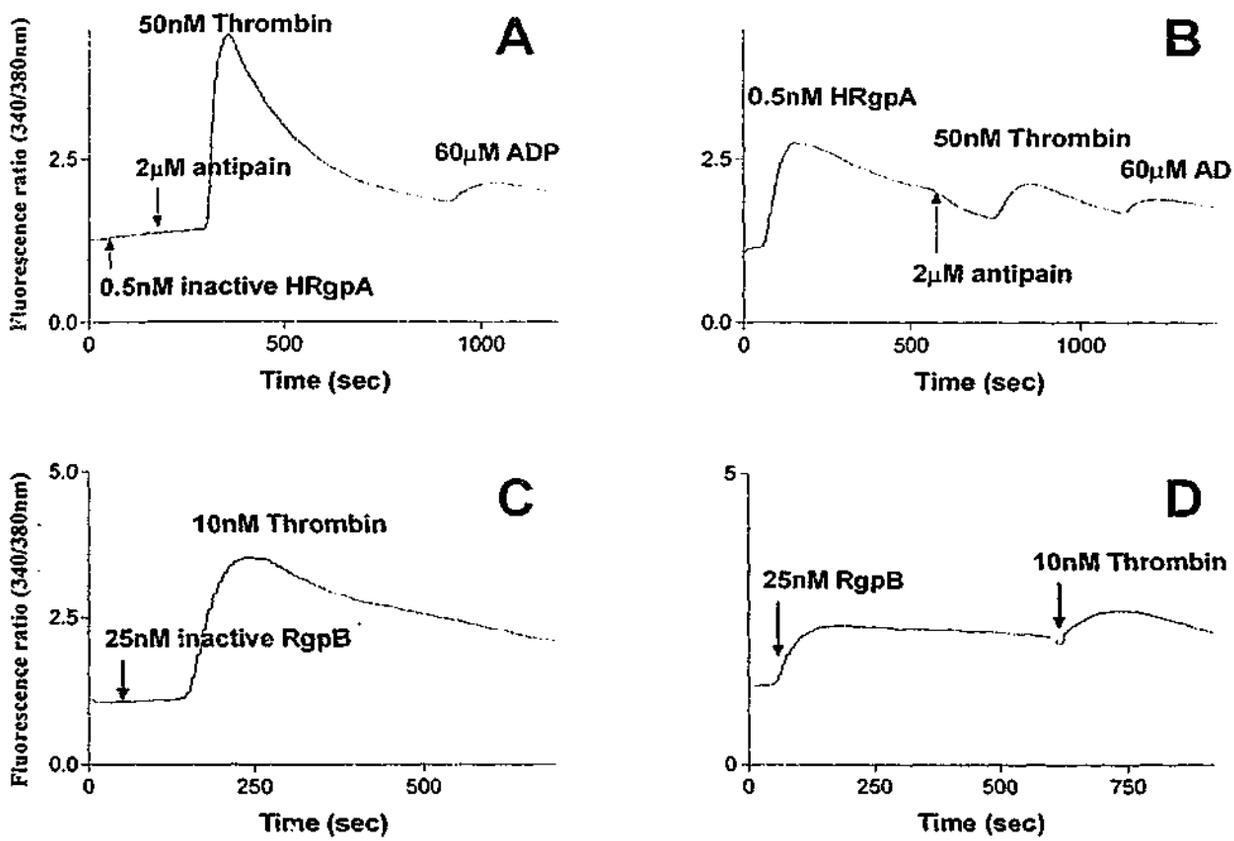


Figure 5.2. **The $[Ca^{2+}]_i$ increase induced in platelets by HRgpA and RgpB is dependent on the proteolytic activity of the enzymes.**

The $[Ca^{2+}]_i$ responses in platelets to: A) 0.5 nM HRgpA inactivated with antipain, or B) 0.5 nM HRgpA, followed by 50 nM thrombin and then 60 μM ADP, C) 25 nM RgpB, or D) 25 nM RgpB inactivated with antipain, followed by 10 nM thrombin. In (A) and (B) antipain (2 μM) was added in between the HRgpA and thrombin in order to ensure that the cysteine protease was not desensitising responses by cleaving thrombin. The $[Ca^{2+}]_i$ responses shown are those for one of the four donors in which these effects were observed.

The question that was then addressed was whether the increase in $[Ca^{2+}]_i$ induced by HRgpA and RgpB activity is due to cleavage of a protease-activated receptor (PAR) on the surface of platelets. Once a PAR is cleaved by a protease it is unable to be activated a second time by the same or another protease in a short period of time. As cleavage of a PAR desensitizes $[Ca^{2+}]_i$ responses to subsequent protease challenges, desensitization of $[Ca^{2+}]_i$ responses by HRgpA, RgpB and thrombin were examined. When platelets were initially activated with HRgpA, a secondary response to this enzyme was virtually eliminated (data not shown). Exposure of platelets to thrombin diminished the $[Ca^{2+}]_i$ response to a second challenge with HRgpA (Fig. 5.3A), whereas treatment of platelets with thrombin or HRgpA did not reduce the response to a subsequent challenge with ADP (Fig. 5.2A &B). The higher the thrombin concentration added to platelets, the smaller was the subsequent HRgpA response (Fig. 5.3A). Similarly, treatment of platelets with HRgpA desensitized the response to a second challenge by thrombin (Fig. 5.3B). As the concentration of HRgpA added to platelets was increased, the subsequent response to thrombin was decreased (Fig 5.3B). Similarly, when platelets were initially exposed to RgpB, a secondary response to this protease or thrombin was abolished and pre-treatment with thrombin desensitized the cells to a subsequent challenge with RgpB (Fig. 5.4). It was shown that the desensitization of the responses was not due to the enzymes inactivating each other. This is illustrated for instance by the finding that adding 2μM antipain (a concentration of inhibitor known to inhibit HRgpA effectively without affecting thrombin) to the cell suspension after the initial activation with HRgpA did not affect the desensitization obtained with thrombin (Fig. 5.2A & B). The addition of the inhibitor after initially adding inactivated HRgpA also did not affect subsequent thrombin responses.

These findings could be extended to all systems tested, strongly indicating that the desensitization of the platelets obtained was purely reflective of prior receptor activation.

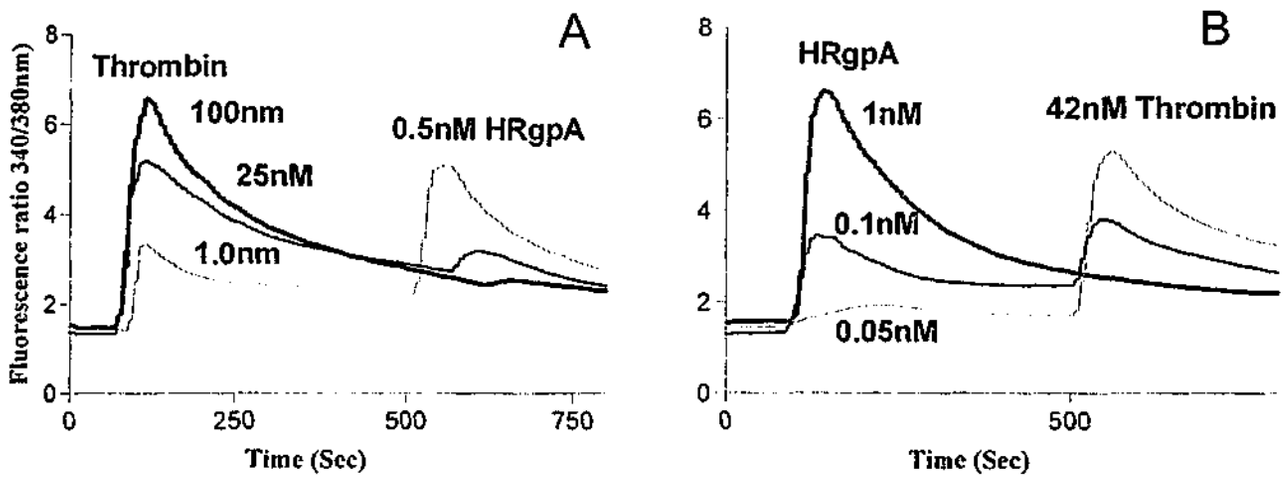


Figure 5.3. **Effects of desensitization of $[Ca^{2+}]_i$ responses in platelets.**
Cells were exposed to: A) 1 nM, 25 nM or 100 nM thrombin, followed by 0.5 nM HRgpA, B) 0.05 nM, 0.1 nM or 1 nM HRgpA, followed by 42 nM thrombin. The results shown represent the desensitization effects for one of the four donors in which these effects were observed.

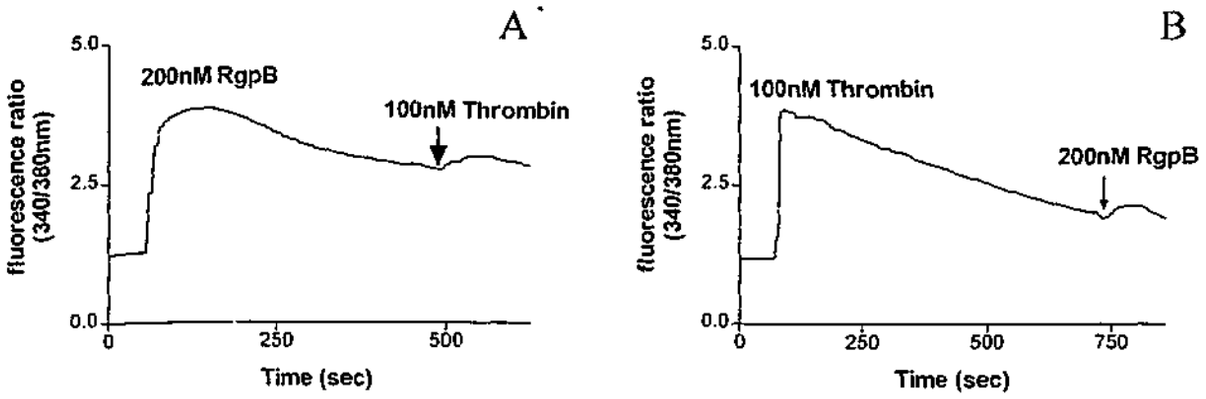


Figure 5.4. **Desensitisation of $[Ca^{2+}]_i$ induced by RgpB and thrombin.**
The $[Ca^{2+}]_i$ responses in platelets to: A) 200 nM RgpB followed by 100 nM thrombin; B) 100 nM thrombin followed by 200 nM RgpB. The $[Ca^{2+}]_i$ responses shown are those for one of the three donors in which these effects were observed.

These desensitization results suggest that HRgpA, RgpB and thrombin activate common receptor(s) on platelets. Thrombin activates PAR-1 and PAR-4 on the surface of human platelets by cleaving after an arginine residue in their extracellular domain. Since HRgpA and RgpB have high specificity of cleavage, limited to peptide bonds after arginine residues, the calcium response observed is probably due to activation of PAR-1 and PAR-4 by these bacterial enzymes. To investigate whether the calcium increase elicited by RgpB and HRgpA is mediated by cleavage of PAR-1, platelets were incubated with rabbit anti-PAR-1 antibody (Jenkins *et al.*, 1993) or a rabbit anti-bromelain antibody (the latter serving as a control) for 15 min, prior to treatment with RgpB or HRgpA. Pre-incubation of platelets with anti-PAR-1 antibody significantly inhibited thrombin and gingipain-induced calcium increases, in comparison to pre-treatment with the control antibody (Fig. 5.5).

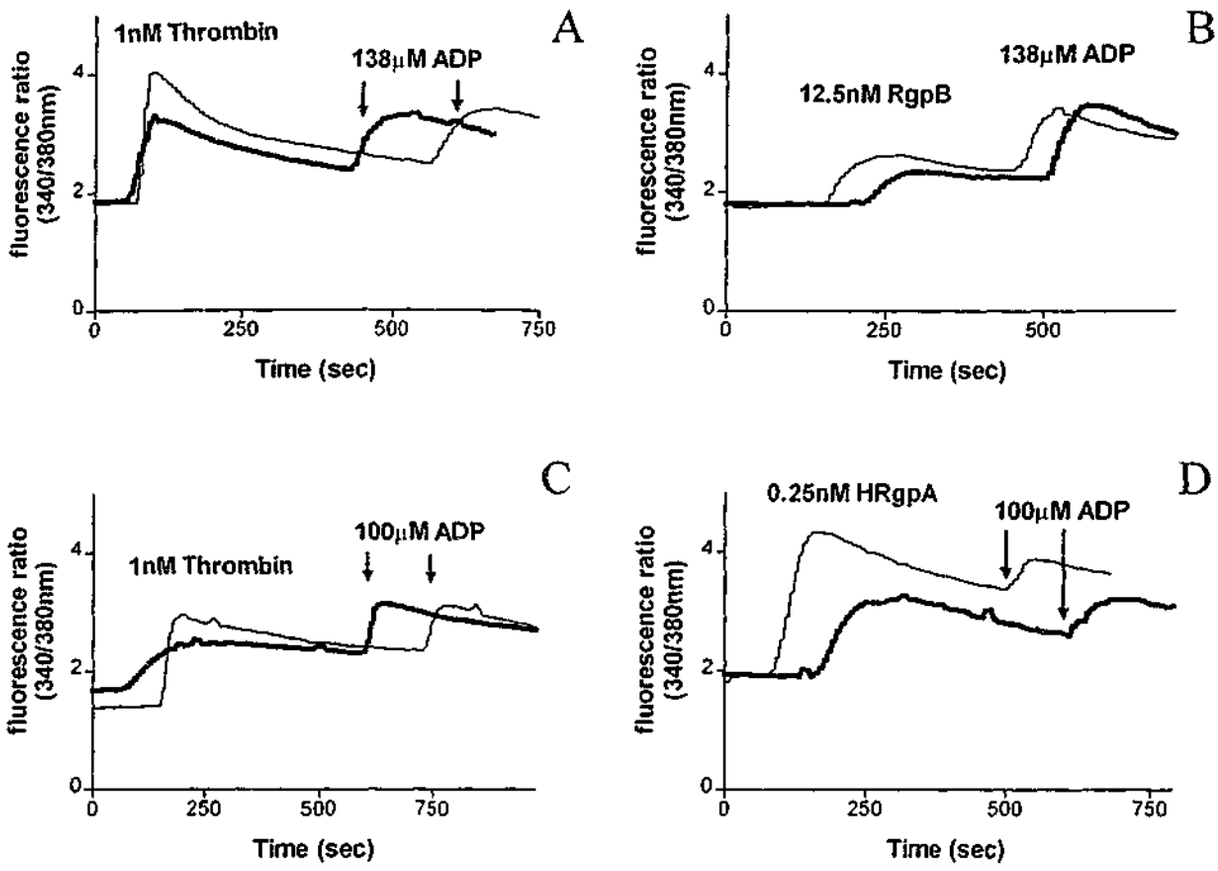


Figure 5.5. **Calcium mobilization in platelets induced by RgpB or HRgpA is reduced by preincubation of cells with PAR-1 antibodies.**

Calcium mobilization in platelets induced by RgpB or HRgpA in the presence of rabbit anti-PAR-1 antibody (——) or control rabbit anti-bromelain antibody (——). Platelets from one donor were incubated with the antibodies (30 μg/ml) for 15 min, before treatment with A) 1 nM thrombin or B) 12.5 nM RgpB, followed by 138 μM ADP. Platelets from another donor were incubated with the antibodies (60 μg/ml) for 15 min, and then treated with C) 1 nM thrombin or D) 0.25 nM HRgpA, followed by 100 μM ADP. These data are representative of three experiments.

Once PARs have been activated by their cognate peptide agonist peptides, they may be rendered desensitized to subsequent exposure to protease agonists, in much the same way as the desensitization described above for treatment with sequential doses of different proteases. Since the agonist peptides for PAR-1 (TRAP – SFRLLN) and PAR-4 (TRAP-4 – AYPGQV) are specific for their cognate receptors in the platelet context, pre-treatment with these peptides may desensitise the cells to subsequent treatment with protease agonists targeting the receptors. As previously reported (Covic *et al.*, 1999; Shapiro *et al.*, 2000) it was found that pre-treatment of platelets with TRAP or TRAP-4 reduced a subsequent $[Ca^{2+}]_i$ response to 1 nM thrombin (Fig. 5.6A&B). Pre-treatment with both peptides virtually abolished a secondary response to thrombin (Fig. 5.6C) and treatment with thrombin reduced a subsequent response to TRAP-4 and abolished the response to TRAP (Fig. 5.6D). As may be seen visually in Fig 5.7 and quantitatively in Table 5.2, pre-treatment of platelets from 3 donors with either TRAP or TRAP-4, individually, markedly reduced the $[Ca^{2+}]_i$ response to 1 nM HRgpA, while treatment with a combination of the peptides all but abolished the response to HRgpA. Pre-treatment of the platelets with HRgpA (Fig. 5.7D) abolished subsequent responses to PAR-4 activating peptide and markedly reduced the response to TRAP. Essentially similar results were found for RgpB (data not shown), demonstrating that the bacterial proteases most likely induce $[Ca^{2+}]_i$ responses in platelets by activating both PAR-1 and PAR-4 receptors.

TRAP caused a rapid increase in $[Ca^{2+}]_i$ followed by a rapid decay, whereas TRAP-4 triggered a more gradual and prolonged increase in $[Ca^{2+}]_i$ (Fig. 5.6 & Fig. 5.7). Previous studies have indicated that PAR-4 signalling is shut off less rapidly than PAR1, probably due to differences in receptor phosphorylation and internalisation (Shapiro *et al.*, 2000; Covic *et al.*, 1999). As seen in Fig. 5.6, following treatment with TRAP-4, thrombin

induced a transient calcium mobilisation similar to the calcium increase caused by TRAP. When platelets pre-treated with TRAP were subsequently exposed to thrombin, the $[Ca^{2+}]_i$ increased and then decreased relatively slowly, similar to the calcium mobilisation induced by TRAP-4. Similar observations were made upon challenge of platelets with HRgpA following pre-treatment with either TRAP or TRAP-4 (Fig. 5.7).

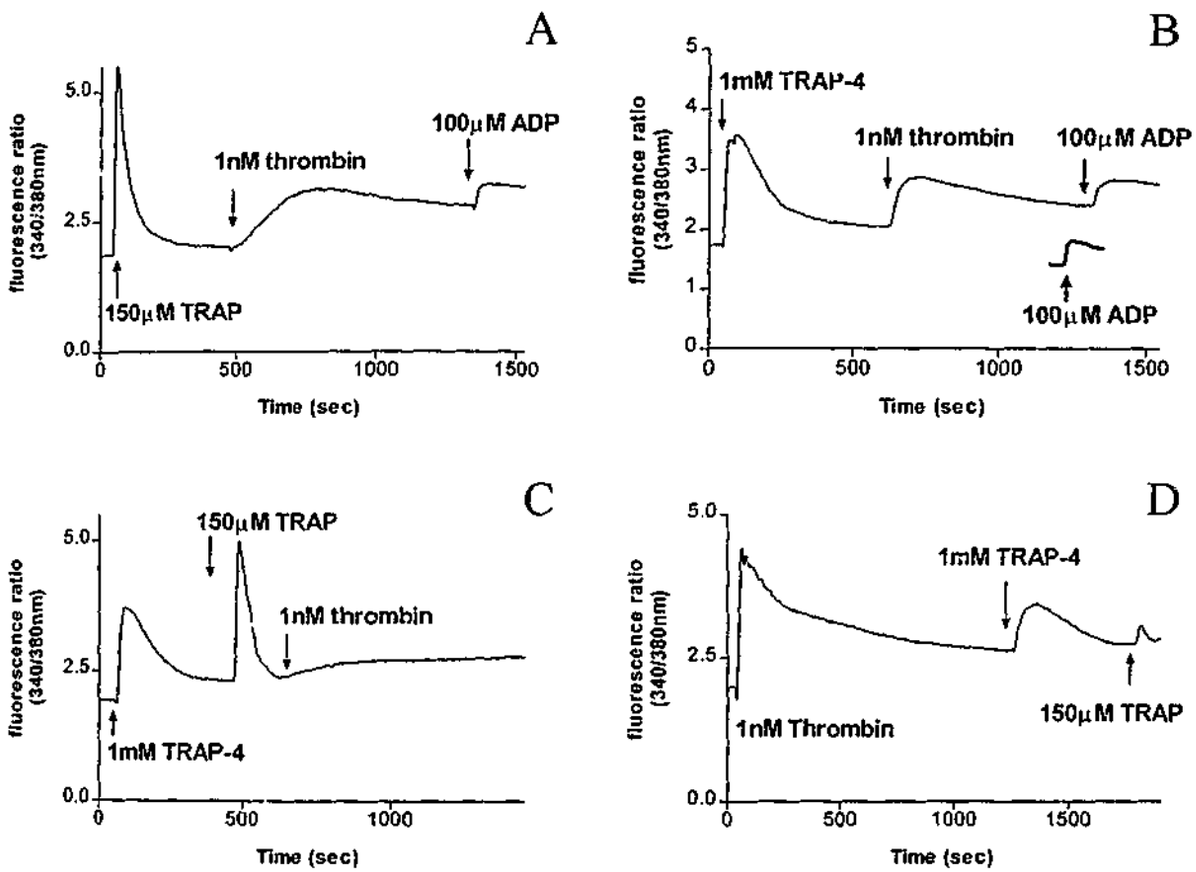


Figure 5.6. **Desensitization of responses to thrombin by prior treatment of platelets with PAR-1 and PAR-4 agonist peptides.**

Calcium responses to 1 nM thrombin following treatment with: A) 150 μM TRAP; B) 1 mM TRAP-4; or C) 1 mM TRAP-4 and 150 μM TRAP. The ADP treatment was used in A & B to demonstrate that prior treatments had not significantly depleted calcium stores, so that essentially normal responses could be obtained with a non-interacting agonist. In (B) the response to ADP prior to any additions to platelets is shown as a bold line for comparison. D) Pre-treatment with thrombin also reduced the response to TRAP-4 and desensitised the response to TRAP.

Table 5.2. **Intracellular calcium responses in human platelets to 1 nM HRgpA following pre-treatment with PAR agonist peptides**

	Fluorescence ratio (340/380nm)			
Donor	**buffer**	**+TRAP-4 (1 mM)**	**+TRAP (150 μM)**	**+TRAP (150 μM) +TRAP-4 (1mM)**
1	4.33	2.19	1.35	NIL
2	3.61	1.58	1.2	0.23
3	3.45	1.02	0.49	NIL

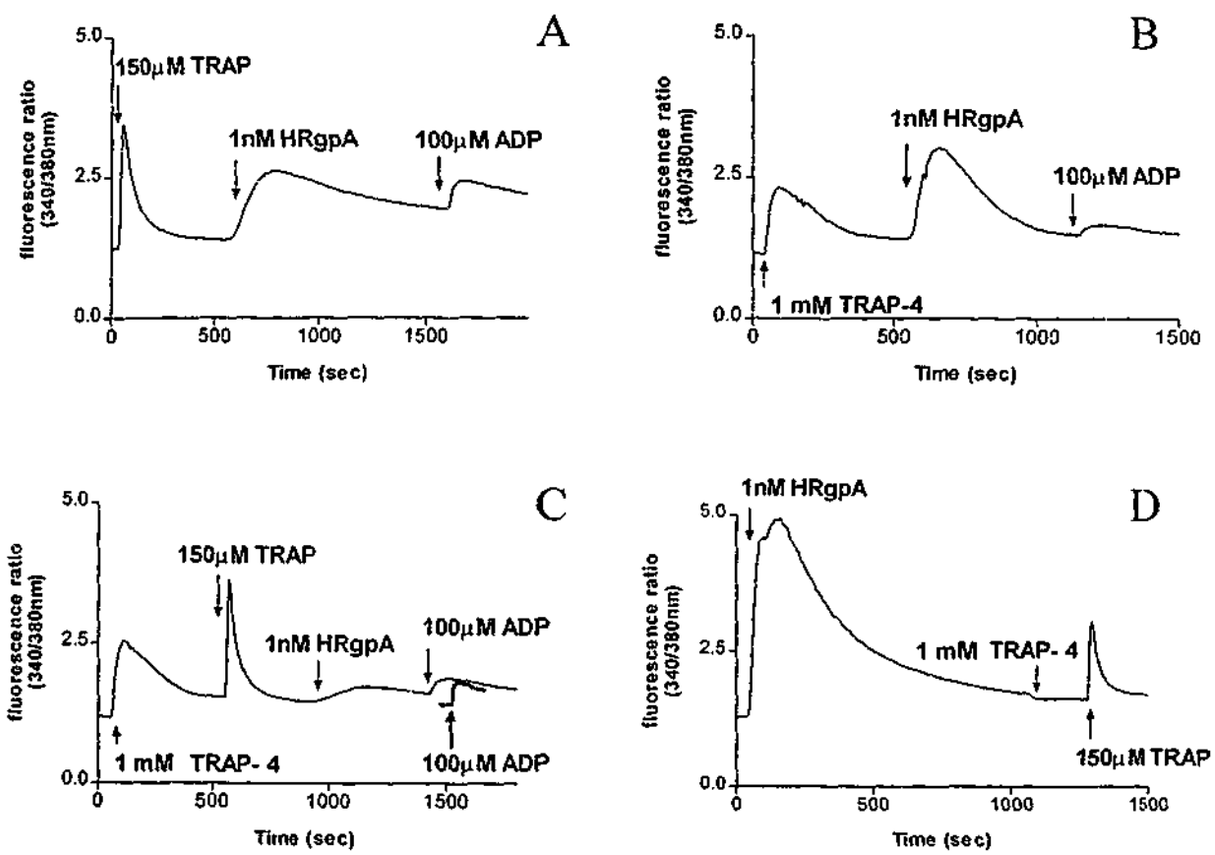


Figure 5.7. **Desensitization of responses to HRgpA by prior treatment of platelets with PAR-1 and PAR-4 agonist peptides.**

Calcium responses to 1 nM HRgpA and 100 μM ADP following treatment with: A) 150 μM TRAP; B) 1 mM TRAP-4; or C) 1 mM TRAP-4 and 150 μM TRAP. The ADP treatment was used in each case to demonstrate that prior treatments had not significantly depleted calcium stores, so that essentially normal responses could be obtained with a non-interacting agonist. In (C) the response to ADP prior to any additions to platelets is shown as a bold line for comparison. D) Pre-treatment with HRgpA also desensitised the response to TRAP-4 and significantly reduced the response to TRAP.

5.1.2 HRgpA and RgpB induce platelet aggregation

Calcium plays a key role in triggering platelet activation and is the single most important intracellular mediator of cell function. To determine whether the $[Ca^{2+}]_i$ elevation induced by the gingipains causes platelet activation, aggregation responses of these cells were investigated. Both HRgpA and RgpB induced aggregation in human platelets, with the rate caused by the same concentration of HRgpA or RgpB differing between cell preparations. The aggregation caused by different concentrations of HRgpA and RgpB in one preparation is shown in Fig.5.8A and B. Much higher concentrations of RgpB were required to cause the same degree of aggregation as that induced by HRgpA.

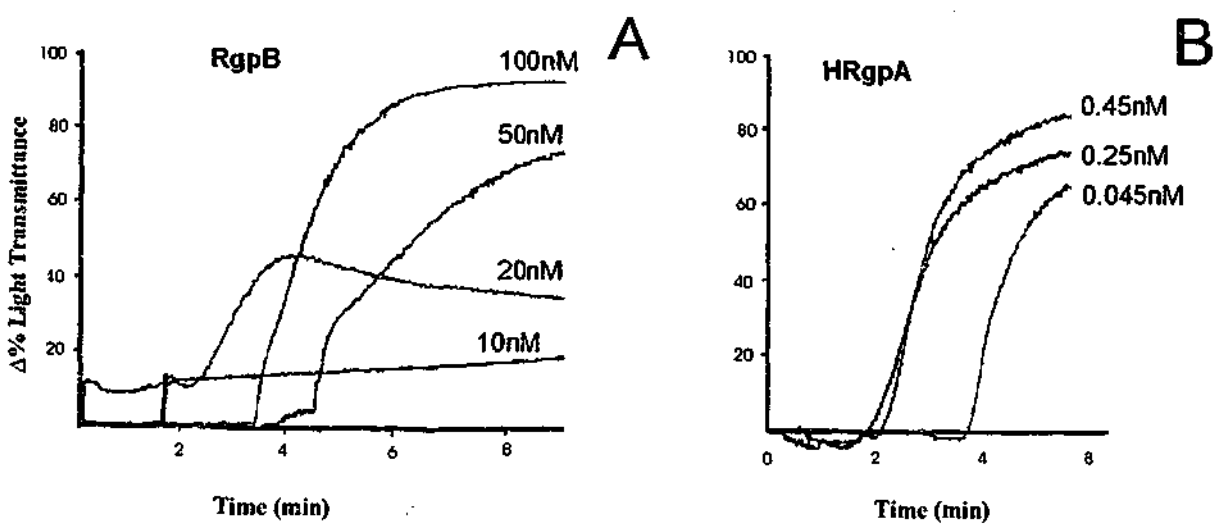


Figure 5.8. **RgpB and HRgpA caused platelet aggregation.**
Platelet aggregation was examined in platelets treated with the indicated concentrations of A) RgpB and B) HRgpA. Traces shown in A and B are from the same donor.

To determine whether the platelet aggregation caused by the bacterial proteases was due to their proteolytic activity, the enzymes were inactivated with 2 μM antipain. This resulted in total inhibition of the aggregation induced by both gingipains (Fig. 5.9A and B), indicating that the cellular effect was due to proteolysis.

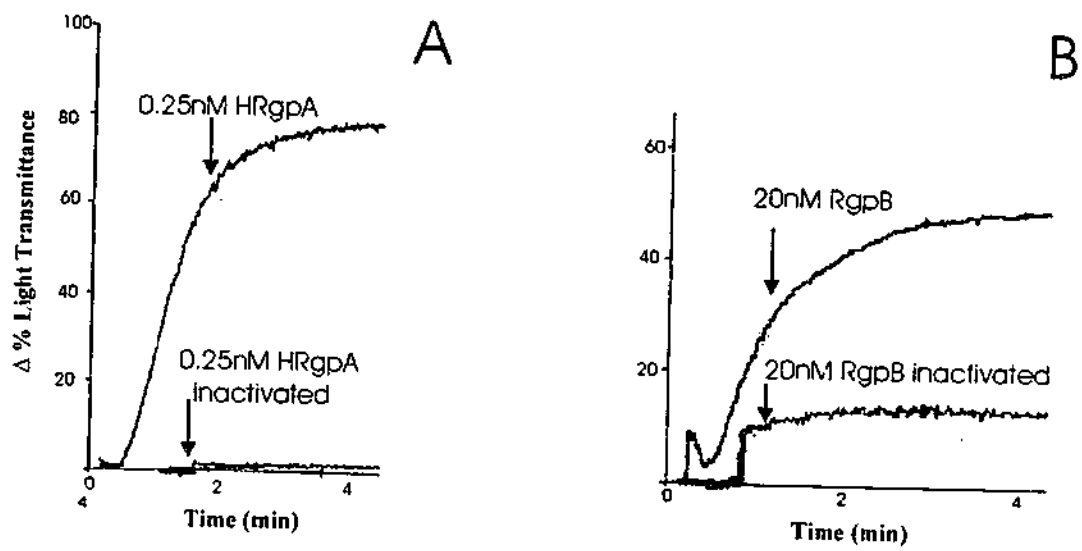


Figure 5.9. **Aggregation of platelets caused by Arg-gingipains is dependent on the proteolytic activity of the enzymes.**

Platelets from healthy volunteers were treated with A) 0.25 nM HRgpA inactivated with antipain or 0.25 nM HRgpA alone; B) 20 nM RgpB inactivated with antipain or 20 nM RgpB alone.

Agglutination of red blood cells by *P. gingivalis* and adherence to other bacteria are thought to mediated at least in part via the proteases of this organism (Curtis *et al.* 1993b, Shi *et al.* 1999). In order to show that the measure of aggregation observed (increase in the light transmittance level) was reflective of true platelet activation and is thus genuine aggregation, rather than an agglutination phenomenon, an inhibitor of platelet activation was used. Pre-incubation of platelets with PGI_2 (100 ng/mL) or forskolin (10 μM) (inhibitors of platelet aggregation) at 37°C for 15 min completely inhibited the aggregation induced by 0.25 nM HRgpA and 20 nM RgpB (Fig.5.10A and B), verifying that HRgpA and RgpB indeed cause true platelet aggregation.

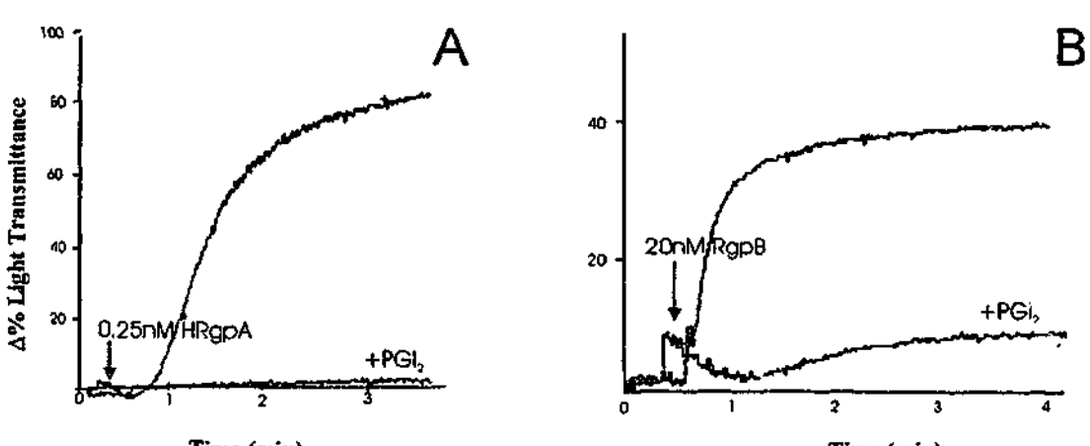


Figure 5.10. **Aggregation induced by HRgpA and RgpB is inhibited by pre-treatment of the cells with inhibitors of platelet activation.**
Platelets from healthy volunteers were pre-treated with PGI_2 or buffer and challenged with A) 0.25 nM HRgpA; B) 20 nM RgpB.

5.1.3 HRgpA and RgpB activate cells stably expressing human PAR-1

In order to determine whether the HRgpA can activate PAR-1 in the same manner as RgpB (Chapter 4), the calcium response elicited by the two enzymes was investigated in transfected N1LF cells stably expressing human PAR-1 (N1LF-PAR-1) in comparison to non-transfected N1LF cells. Treatment of N1LF-PAR-1 cells with 150 μM TRAP (Fig. 5.10B), or 10 nM thrombin (Fig. 5.11D), elicited an intracellular $[Ca^{2+}]_i$ increase, while non-transfected N1LF cells did not respond to 150 μM TRAP (Fig. 5.11A), or to 20 nM thrombin (Fig. 5.11C). Concentrations of HRgpA as low as 2 nM elicited a $[Ca^{2+}]_i$ increase in N1LF PAR-1 cells, whereas concentrations of the enzyme as high as 100 nM did not cause calcium mobilization in non-transfected N1LF cells (data not shown). Treatment with 18 nM HRgpA caused an intracellular $[Ca^{2+}]_i$ increase in N1LF PAR-1 cells (Fig. 5.11F). In contrast, non-transfected N1LF cells did not respond to 18 nM HRgpA but did respond to bradykinin, showing that G-protein-coupled receptor signalling pathways were intact (Fig. 5.11E). Thus, HRgpA activates PAR-1.

As shown in Chapter 4 exposure of N1LF-PAR-1 cells to 18nM RgpB induced an intracellular $[Ca^{2+}]_i$ increase (Fig. 5.11H), whereas non-transfected N1LF cells did not respond to 18nM RgpB (Fig. 5.11G) but did respond to bradykinin, verifying that RgpB can indeed activate PAR-1. Both HRgpA and RgpB induced a dose-dependent increase in $[Ca^{2+}]_i$ in N1LF-PAR-1 cells. The concentration dependence of such an increase is shown in Fig. 5.12, yielding an enzyme concentration inducing half the maximal response (EC_{50}) of 17 nM, 48 nM and 0.26 nM for receptor activation by HRgpA, RgpB and thrombin, respectively.

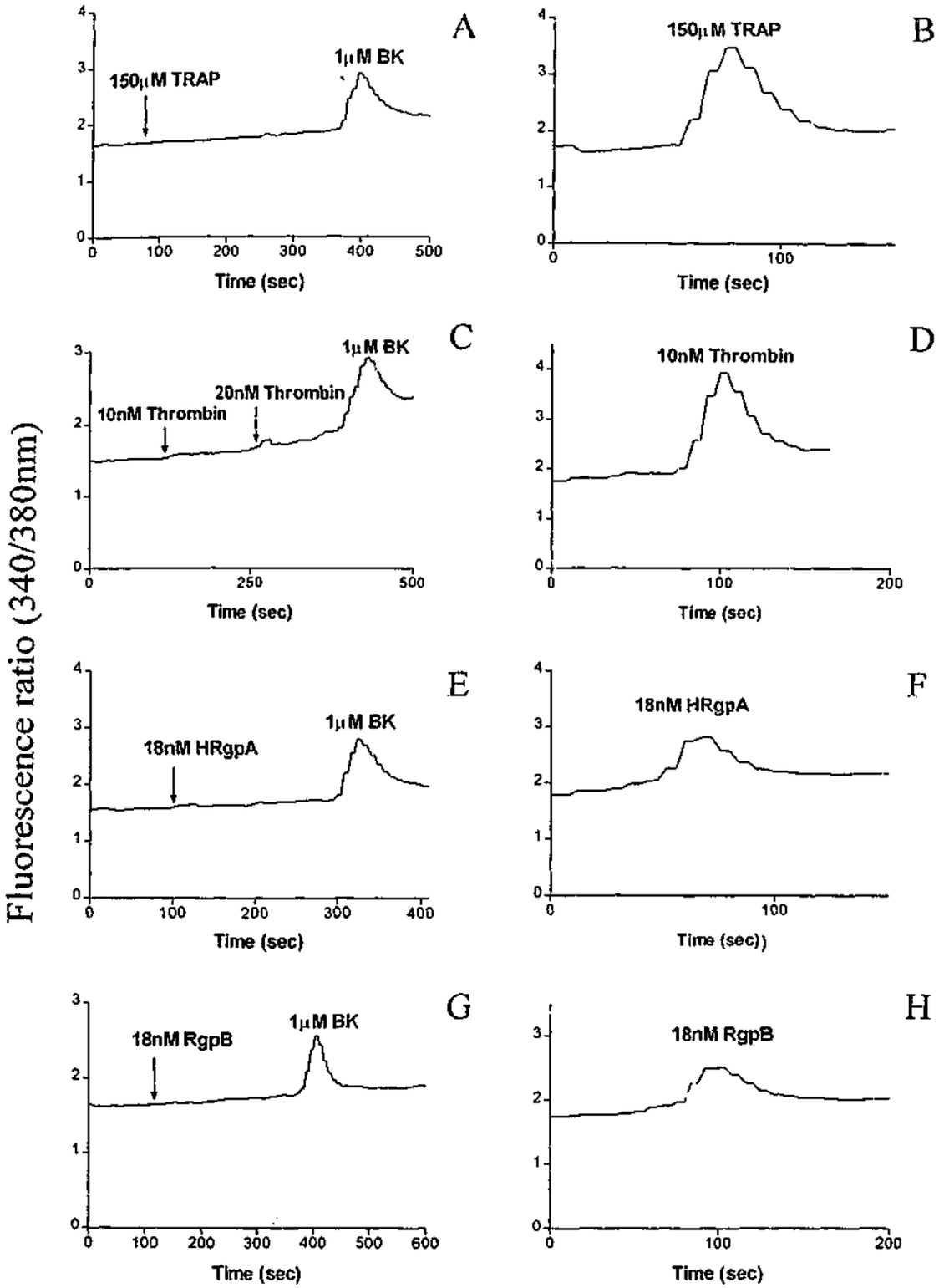
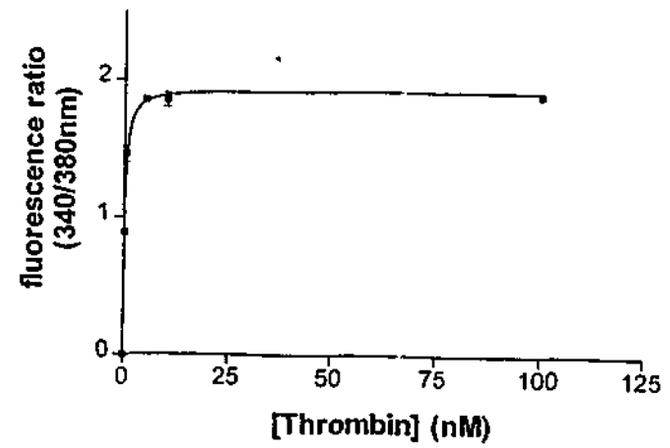
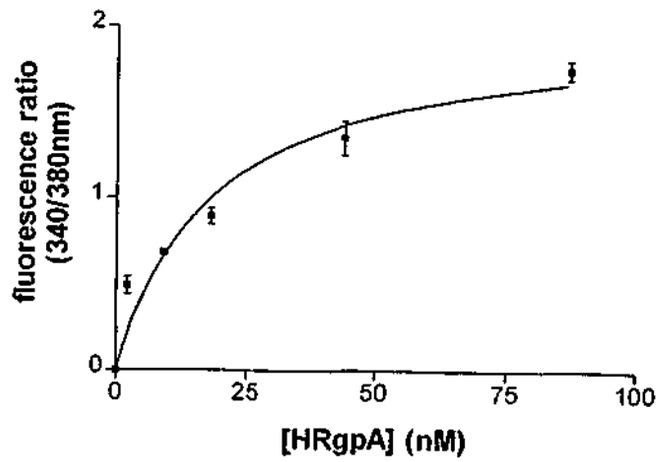


Figure 5.11. **Arg-gingipains activate PAR-1 on transfected cells stably expressing human PAR-1.** Calcium mobilization in N1LF cells (A, C, E, G) or N1LF PAR-1 cells (B, D, F, H) induced by (A, B) 150 μM TRAP; (C, D) 10 nM thrombin; (E, F) 18 nM HRgpA and (G, H) 18 nM RgpB. N1LF cells responded to 1 μM bradykinin in each case. The traces are representative of three similar sets of data obtained.





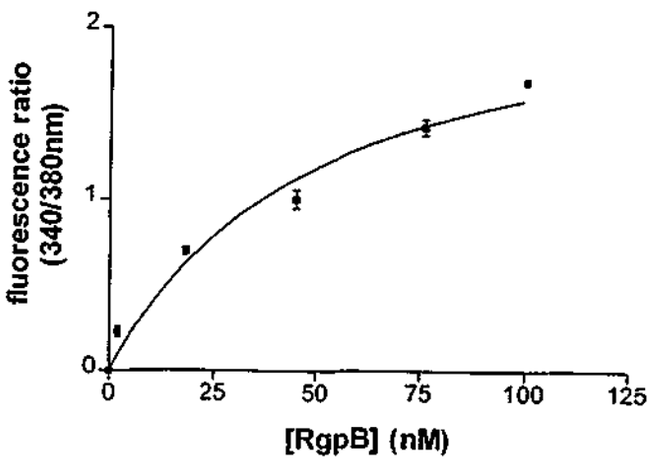


Figure 5.12. **The $[Ca^{2+}]_i$ response of N1LF PAR-1 cells to increasing concentrations of thrombin, HRgpA and RgpB.**

5.2.4 HRgpA and RgpB activate cells stably expressing human PAR-4

To investigate whether HRgpA and RgpB can cleave and activate PAR-4, the calcium response elicited by these two enzymes was studied in transfected N1LF cells stably expressing human PAR-4 (N1LF-PAR-4) in comparison to non-transfected N1LF cells. The cells were loaded with Fura-2 and $[Ca^{2+}]_i$ responses to thrombin, HRgpA, RgpB and PAR-4 agonist peptide (TRAP-4) were measured.

Treatment of N1LF-PAR-4 cells with 100 μM TRAP-4 or 500 μM TRAP-4 (Fig. 5.13B) or 25nM thrombin (Fig. 5.13D) elicited an intracellular $[Ca^{2+}]_i$ increase, while non-transfected N1LF cells did not respond to 500 μM TRAP-4 (Fig. 5.13A) or 20 nM thrombin (Fig. 5.13C). The calcium response induced in N1LF-PAR-4 cells by 4.5 nM HRgpA and 18 nM RgpB is shown in Figs. 5.13F & H, compared to the lack of response in non-transfected N1LF cells (Fig. 5.13E & G). Concentrations of HRgpA as low as 0.45 nM elicited a $[Ca^{2+}]_i$ increase in N1LF-PAR-4 cells (data not shown).

Both HRgpA and RgpB induced a dose-dependent increase in $[Ca^{2+}]_i$ in N1LF PAR-4 cells, verifying that these enzymes can activate PAR-4. The concentration dependence of such an increase is shown in Fig. 5.14, yielding an enzyme concentration inducing half the maximal response (EC_{50}) of 1.7 nM, 4.6 nM and 10 nM for receptor activation by HRgpA, RgpB and thrombin, respectively.

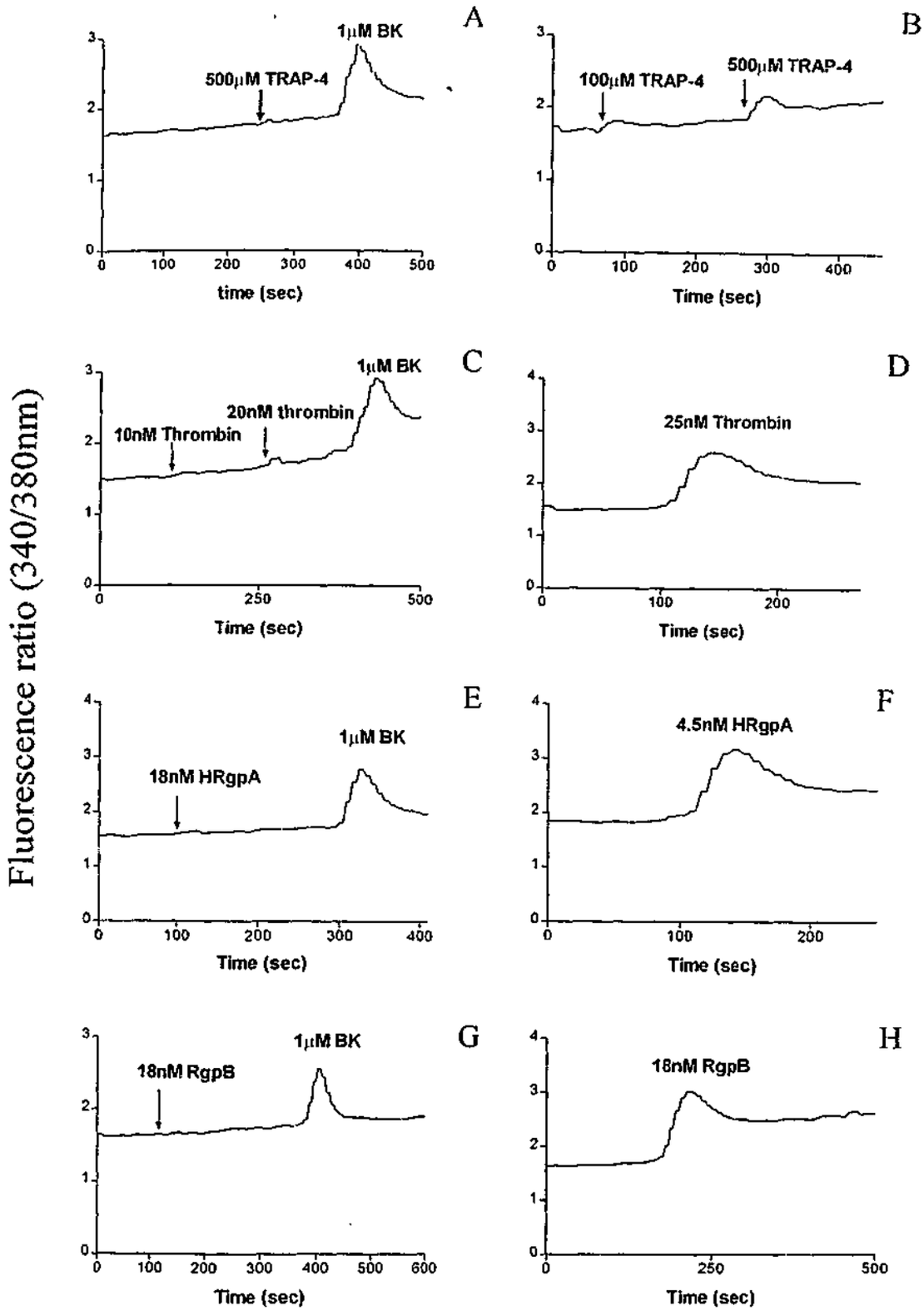


Figure 5.13. **Arg-gingipains activate PAR-4 on transfected cells stably expressing human PAR-4.** Calcium mobilization in N1LF cells (A, C, E, G) or N1LF PAR-4 cells (B, D, F, H) induced b (A, B) 100 μM or 500 μM TRAP-4 ; (C, D) 25 nM thrombin; (E, F) 18 nM or 4.5 nM HRgpA and (G, H) 18 nM RgpB. N1LF cells responded to 10 nM bradykinin in each case. The traces are representative of three similar sets of data obtained.

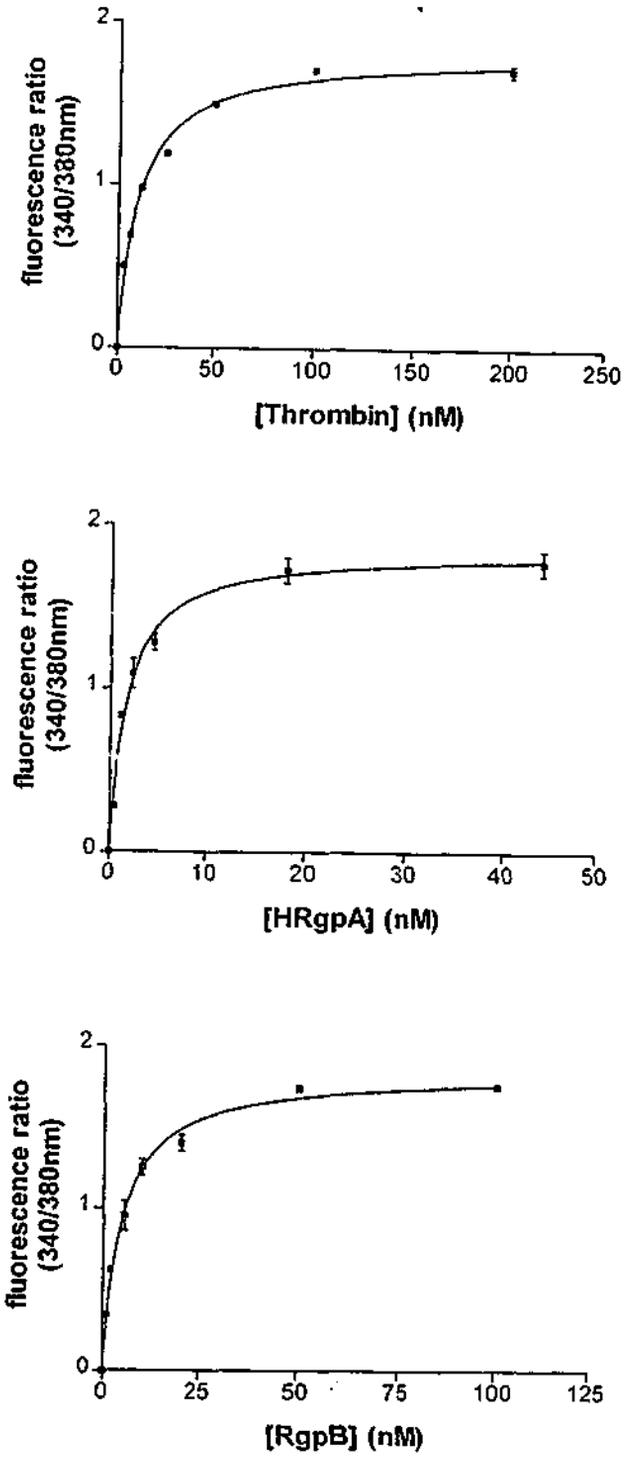


Figure 5.14. **The $[Ca^{2+}]_i$ response of N1LF PAR-4 cells to increasing concentrations of thrombin, HRgpA and RgpB.**

5.3 Discussion

An emerging theme in the interaction between pathogenic bacteria and the host is the ability of the microbial invader to proteolytically modify the host cell surface proteins, including various receptors. In most cases, bacterial proteases degrade receptors (Ijiri *et al.*, 1994; Lala *et al.*, 1994; Jagels *et al.*, 1996; Sugawara *et al.*, 2000), or release soluble ectodomains (Vollmer *et al.*, 1996; Wolf *et al.*, 1994) leading to desensitization of cellular responses to their physiological agonist. Such treatment of cells of the immune system may potentially affect antimicrobial defence mechanisms and/or interfere with the regulation of the inflammatory reaction. These effects can be even more deleterious if receptors such as the PARs are the target of bacterial proteases, since cleavage of these receptors will lead to uncontrolled activation of host cells which, in the case of platelets, is normally mediated by tightly regulated thrombin cleavage of PAR-1 and PAR-4. However, despite the fact that unchecked platelet activation may have serious pathological consequences if bacteria possessing such an ability find their way into the blood stream, the interaction between platelets and proteases from pathogenic bacteria has never been systematically investigated.

An example of bacteria that are known to escape into the blood stream on a regular basis are members of the microbial flora forming dental plaque. This ability correlates with the severity of periodontitis when even such simple activities as chewing, flossing and brushing cause a transient bacteraemia (Silver *et al.*, 1980; Carroll & Sebor 1980; Sconyers *et al.*, 1973). Fortunately, most of the bacteria are benign oral streptococci, which can cause a serious medical problem (endocarditis), only in subjects with damaged or implanted heart valves (Durack and Phil, 1995; Van de Meer *et al.*, 1992). In the case of severe periodontitis, however, *P. gingivalis* can also find its way into the circulation and even

infect atherosclerotic plaques (Haraszthy *et al.*, 1998; Chiu, 2000). Thus, the effect of arg-specific gingipains on platelets was closely investigated.

HRgpA and RgpB induce an increase in $[Ca^{2+}]_i$ in human platelets which is dependent upon their proteolytic activity. Several lines of evidence strongly support the hypothesis that this increase is mediated by activation of PAR-1 and PAR-4. Exposure of human platelets to either of the gingipains desensitized the $[Ca^{2+}]_i$ response to a second challenge with the same enzyme, a phenomenon that is in agreement with the rapid desensitization that protease-activated receptors undergo after a short period of activation with a protease. Desensitization studies carried out with thrombin showed that when human platelets were activated with this enzyme, a secondary challenge with HRgpA or RgpB was virtually eliminated. Similarly, pre-treatment of platelets with the bacterial enzymes desensitized the response to a second challenge with thrombin, suggesting that both enzymes activate the platelet thrombin receptors. Antibodies specific for the sequence which occurs immediately amino–terminal to the cleavage site of PAR-1 were able to decrease the $[Ca^{2+}]_i$ increase caused by HRgpA or RgpB by approximately 50%. This finding supports the proposed mechanism that $[Ca^{2+}]_i$ mobilization by these proteases is in part mediated by cleavage of PAR-1. The ability of the bacterial proteases to activate the receptors was further investigated by using the individual agonist peptides for PAR-1 and PAR-4 to desensitise a subsequent response to the gingipains. For each of the enzymes, it was shown that prior exposure of the platelets to a combination of the PAR-1 and PAR-4 agonist peptides almost completely abolished the response to the protease. This provides strong evidence that the bacterial proteases induce responses in platelets by cleaving and activating both PAR-1 and PAR-4. Interestingly, exposure of cells to 1 nM thrombin abolished a subsequent response to TRAP but only reduced a secondary response to

TRAP-4, suggesting that 1 nM thrombin activates mainly PAR-1 whereas PAR-4 can still be activated by its agonist peptide. This is in agreement with previous studies (Kahn *et al.*, 1999; Covic *et al.*, 2000) that have shown that PAR-1 on human platelets mediates responses to low concentrations of thrombin. Pre-treatment of platelets with 1 nM HRgpA abolished a subsequent response to TRAP-4 but only reduced a secondary response to TRAP, suggesting that 1nM HRgpA efficiently activates PAR-4 whereas PAR-1 can still be activated by its agonist peptide.

Thrombin activates PAR-1 and PAR-4 expressed on the surface of platelets by cleaving after a specific arginine residue at their respective activation sites. HRgpA and RgpB, which are absolutely specific for hydrolysis at Arg-Xaa sites, were found to activate both PAR-1 and PAR-4 in transfected cells stably expressing these receptors. HRgpA cleaved PAR-4 approximately six times more efficiently than thrombin, based on evaluation of the EC_{50} in PAR-4 transfected cells, while RgpB was approximately 2-fold more efficient. Thrombin was a much more efficient activator of PAR-1, however, activating the receptor 60 times more efficiently than HRgpA and 160 times more efficiently than RgpB. The greater efficiency of thrombin for cleavage of PAR-1 is expected in the light of the specific association of the protease with a hirudin-like sequence that binds its fibrinogen binding exosite (Vu *et al.*, 1991b; Liu *et al.*, 1991). PAR-4 does not have a hirudin-like domain and this accounts for its less effective cleavage by thrombin compared to PAR-1, as seen in the present study and described elsewhere (Kahn *et al.*, 1998a; Xu *et al.*, 1998). The gingipains would also not be expected to cleave PAR-1 very efficiently in comparison to thrombin since they presumably have no mechanism for additional interactions with the receptor that are analogous to thrombin.

HRgpA exhibited higher efficiency in activating both PAR-1 and PAR-4 compared to RgpB. It has previously being found that the proteolytic activity of HRgpA against azocasein or azocoll was five times higher than that of RgpB, using equimolar amounts of HRgpA and RgpB, despite the fact that both enzymes were equally active on synthetic substrates (Potempa *et al.*, 1998). Similarly, HRgpA has been found to have higher activity than RgpB in activating factor X (Imamura *et al.*, 1997). Both HRgpA and RgpB were more efficient at cleaving PAR-4 compared to PAR-1. Although these enzymes are highly specific for cleavage after arginine residues, less is known about the effect of amino acid residues around the cleavage site on the catalytic potency of HRgpA and RgpB. In one study, the specificity against synthetic substrates with a P_1 Arg residue was investigated and was found to be similar for HRgpA and RgpB, and no clear preference was observed for particular amino acid residues at the P2 and/or P3 position (P3-P2-Arg) (Potempa *et al.*, 1998). Elucidation of the crystal structure of RgpB revealed that, with the exception of the entrance hole to the S1 pocket, which is optimised to accommodate arginine side chains, the molecular surface around the active site of the enzyme is very flat and carries a negative electrostatic potential (Eichinger *et al.*, 1999). It is thought that this open binding site and strong binding of the Arg residue enables RgpB to cleave a multitude of Arg-X bonds in proteins and peptides. HRgpA is comprised of RgpA noncovalently complexed with adhesins. As the structure of HRgpA has not been elucidated, the role of adhesins in the structure and activity of the enzyme is not known. It may be postulated that the adhesin subunits could affect the interaction of the protease with the surface of the cell by serving as an anchor and stabilizing this interaction. More stable interaction of the enzyme with the cell surface may contribute to more efficient cleavage of protease-activated receptors.

Based on the desensitization studies and the ability of HRgpA and RgpB to activate PAR-1 and PAR-4, it can be concluded that these bacterial proteases activate these receptors on the surface of platelets, resulting in calcium mobilization. Treatment of human platelets with HRgpA or RgpB also resulted in platelet aggregation, which was dependent upon the proteolytic activity of the enzymes. In platelets treated with either gingipain, the increase in light transmittance level observed was a true aggregation phenomenon since pre-treatment of platelets with PGI_2, an inhibitor of platelet aggregation, prevented aggregation. The aggregation assay was carried out in the absence of exogenously added fibrinogen. In thrombin-induced platelet aggregation, fibrinogen is released from the α-granules during platelet activation, which then binds to the GP IIb-IIIa complex, resulting in aggregation (Fox, 1993). HRgpA and RgpB cause aggregation without the requirement of exogenous fibrinogen, indicating that fibrinogen is released as part of platelet activation by these bacterial enzymes.

HRgpA caused a $[Ca^{2+}]_i$ increase in platelets and aggregation at much lower concentrations than RgpB. This would be expected in view of the fact that HRgpA not only showed much higher efficacy than RgpB in activating PAR-1 and PAR-4 in cells stably expressing these receptors, but also was a more potent activator of PAR-4 than thrombin. HRgpA activates platelets with similar efficiency to thrombin, and this may arise from its more potent cleavage of PAR-4 as seen in the transfected cells. In human platelets, PAR-4 mRNA has been detected at ~30% of PAR-1 mRNA levels (Kahn *et al.* 1999). Based on studies using blocking antibodies, peptides that inhibit activation of PAR-1 and PAR-4 and PAR agonist peptides it appears that PAR-1 on human platelets responds to low concentrations of thrombin and PAR-4 mediates responses to high concentrations of thrombin (Kahn *et al.*, 1999; Covic *et al.*, 2000). It has also shown that

activation of PAR-4 alone, using TRAP-4 peptide, is sufficient to cause platelet activation (Covic *et al.*, 2000; Kahn *et al.*, 1999). HRgpA, by potently activating PAR-4 and at the same time activating the more abundant PAR-1 receptor, may be causing the same overall effect induced by thrombin.

The data discussed above present compelling evidence that PAR-1 and PAR-4 on the platelet surface can be activated by Arg-Xaa specific bacterial proteases. Recently, periodontal diseases have been linked to cardiovascular illnesses, including heart attack, in a number of epidemiological studies (Page, 1998). The consensus viewpoint is that this correlation is an effect of sustained chronic inflammation (Beck, 1996; Loesche, 1994; Mombelli and Weyer, 1998; Joshipura *et al.*, 1998; Lopes-Virella & Virella 1985) of the periodontium triggered by continuous release of LPS from gram negative bacteria. In this scheme, *P. gingivalis* proteases would have an indirect role as factors aggravating and/or sustaining chronic inflammation. In light of the data presented here, however, it is tempting to speculate that gingipains-R may have a more direct role in cardiovascular complications. Gingipains-R may cause platelet aggregation during bacteraemias, resulting in the production of thrombi that possibly occlude coronary arteries with cardiovascular implications. Recently, *P. gingivalis* has been immunolocalized in the shoulders of atherosclerotic plaque (Chiu, 2000). If these bacterial cells still express gingipains, it is very likely that such proteases will contribute to plaque ulceration and thrombus formation through effective, uncontrolled activation of both coagulation factors and PARs.

CHAPTER 6

Final Discussion

Cellular microbiology is a new research field that investigates "the silent, biochemical conversation between the microbe and its host" (Fasano, 1999). Eukaryotic cells communicate with their environment as molecules, eg. hormones, outside the cell bind to cell surface receptors, generating a signal that is then relayed through intracellular G-proteins to induce a cellular response. Several pathogenic bacteria produce molecules that have also been found to activate receptors expressed on the surface of human cells and thus elicit intracellular signals. Enterotoxigenic *Escherichia coli*, which causes enteric disease in humans produces heat-stable enterotoxins (STs) (Giannella, 1995). ST_a is a small peptide that activates a member of the guanylate cyclase receptor family and thus causes an increase in the intracellular concentration of cGMP (Visweswariah *et al.*, 1994), which activates apical chloride secretion (Giannella, 1995) resulting in secretory diarrhea. Similarly, Raimondi *et al.* (1995) demonstrated that the enterotoxic effect of the thermostable direct haemolysin elaborated by *Vibrio parahaemolyticus* was mediated by modulation of the intracellular calcium concentration, which is another regulator of electrolytes in intestinal cells (Donowitz, 1994). Hemolysin stimulates intracellular calcium increase by interacting with a polysialoganglioside GT1b surface receptor and thus induces intestinal chloride secretion. Thus, enteric pathogens acting through host receptors can induce intestinal electrocyte secretion and subsequent diarrhea although the role this plays in bacterial pathogenesis is not well understood.

In the present study the proteolytic enzymes gingipains-R produced by the periodontal pathogen *P. gingivalis* were found to interact with human protease activated receptors (PARs). The ability of gingipains-R to cleave and activate human PAR-1, -2 and -4 was most conclusively demonstrated using cells stably expressing these receptors (Chapter 3, 4, 5). It was shown that gingipains-R induced intracellular calcium mobilization in transfected cells expressing PAR-1, -2 and -4 but not in control cells not expressing these receptors. PARs are thought to play a role in coagulation and inflammation (Coughlin, 2000), as they are expressed on cells involved in these processes and thus have potential roles in disease. Periodontal disease is an inflammatory condition with massive neutrophil infiltration into the gingiva and high levels of pro-inflammatory cytokines in this tissue and the gingival crevicular fluid. This disease condition has been linked to atherosclerosis and cardiovascular disease.

The effect of gingipains-R on human cells, which express PARs and can contribute to the inflammatory process in periodontal disease, was evaluated. It was found that treatment of human neutrophils by RgpB immediately caused an intracellular calcium increase, which was dependent on its proteolytic activity and calcium desensitisation studies indicated that RgpB activates PAR-2 (Chapter 1). Treatment of neutrophils with RAP, trypsin or RgpB did not cause myeloperoxidase release indicating that PAR-2 activation does not stimulate neutrophil degranulation. In aggreement with a previous study (Howells *et al.*, 1997) it was shown that activation of PAR-2 causes an increase in the expression of CD11b, a subunit of the adherence receptor, on the surface of neutrophils and can therefore lead to neutrophil adherence and migration into inflamed tissue. RgpB was found to be a weaker agonists than RAP in inducing CD11b up-regulation in the donors tested. Due to the variability of the response to both RAP and RgpB it is necessary to

examine the effects of these agonists in a larger number of donors. There is growing evidence that neutrophils can produce a number of cytokines such as Interleukin-1α (IL-1α), IL-8, IL-12 and Tumor Necrosis Factor-α (Cassatella *et al.*, 1999). A wide range of stimuli can cause expression of cytokines by neutrophils, including activation of the G-protein-coupled receptors for fMLP and C5a (Cassatella *et al.*, 1999). It would thus be of interest to examine whether activation of -the G-protein-coupled receptor- PAR-2 by its agonist peptide or RgpB can affect cytokine production in neutrophils.

RgpB also caused an increase in intracellular calcium levels in KB60 cells, an oral epidermioid cell line that has been extensively used as a model to study gingival epithelial cells. KB60 cells were found to express PAR-1, -2 and -3 and the calcium mobilisation induced by RgpB was dependent on the proteolytic activity of the enzyme. Based on the ability of RgpB to activate PAR-1 and PAR-2, the desensitization studies carried out strongly suggest that this bacterial enzyme activates both PAR-1 and PAR-2 in KB60 cells (Chapter 4). Treatment of KB60 cells with RgpB, thrombin, trypsin, TRAP or RAP increased the secretion of the pro-inflammatory cytokine IL-6. IL-6 is one of the pro-inflammatory cytokines that are present in the gingival crevicular fluid and in the gingiva of patients with periodontal disease. Thus, *P. gingivalis* through its proteases may actually make use of host cell receptors to exacerbate the inflammatory process that characterises periodontal disease. Further investigations are required to validate this hypothesis including determination of expression of PARs in gingival epithelial cells *in vivo* and examination of whether PAR activation by agonist peptides and gingipains-R leads to up-regulation of IL-6 and other pro-inflammatory cytokines.

HRgpA and RgpB were found to induce calcium mobilisation in human platelets and this effect was dependent on their proteolytic activity. Desensitisation studies with thrombin and PAR agonist peptides in combination with the finding that gingipains-R can activate PAR-1 and -4 in cells expressing the receptors, allow the conclusion that gingipains-R are activating PAR-1 and -4 in human platelets (Chapter 5). Furthermore, gingipains-R, through their proteolytic activity, induced platelet aggregation, which is consistent with PAR-1 and PAR-4 activation on human platelets. The reported presence of *P. gingivalis* cells in atherosclerotic plaques allows the suggestion that if these bacterial cells still express gingipains they may indeed affect platelets. Further work is necessary to determine whether gingipain-R-platelet interaction is likely to occur *in vivo*. Following intravenous infusion of *P. gingivalis* and/or gingipains-R into an animal model, it is necessary to investigate whether bacterial cells or their products interact with circulating platelets resulting in the formation of platelet vegetations and thromboemboli.

Activation of human PARs by gingipains-R from *P. gingivalis* is a new paradigm in microbial pathogenicity, ie. that some host cell functions may be manipulated by bacterial proteases cleaving the PARs. In the case of *P. gingivalis*, the immediate and evolutionary advantage of this new pathway for the pathogen may not be clearly apparent. However, uncontrolled PAR activation will certainly contribute to the deregulation of the local inflammatory reaction, which can be beneficial for the microbial community in the pathological periodontal pocket. It can be postulated that gingipains-R by activating PARs may stimulate neutrophils and cause gingival epithelial cells to secrete pro-inflammatory cytokines, and thus contribute to the local inflammatory reaction within the pathological periodontal pocket and the propagation of the chronic inflammatory condition present in periodontal disease. The platelet aggregation and possible subsequent thrombus formation

induced by Rgps may be advantageous for *P. gingivalis*, as it may serve to entrap red blood cells thus providing a source of heme iron, which is essential for the bacterium's growth. Platelets play an important role in acute arterial thrombosis, thus gingipains-R, by activating PARs on human platelets and causing platelet aggregation, may promote thrombosis and therefore provide a molecular mechanism underlying the reported association between periodontal disease and cardiovascular disease. Activation of PARs by these bacterial proteases may also provide a broader link in the mechanism by which a bacterially induced inflammatory condition can affect coagulation. PARs are also expressed on vascular endothelial cells and their activation has been found to induce vascular permeability and up-regulation of adhesion molecules that may promote adhesion of platelets and leukocytes to the vascular endothelium. Following bacteremia, *P. gingivalis* and gingipains-R may come in contact with vascular endothelial cells and through PAR activation further promote inflammation, vascular injury and thrombosis. In order to test this hypothesis, it would be necessary to examine the interaction of Rgps with PARs on human endothelial cells *in vitro* and determine the effect of Rgps on vascular endothelial cells in an animal model of infection. Gingipains-R in addition to the soluble forms used in this study are also present in membrane bound forms which are present on the cell surface of whole bacteria and blebs of the outer membranes called vesicles. Preliminary studies have shown that *P. gingivalis* vesicles are also capable of activating PAR-1, -2, -4 and inducing calcium mobilization in KB60 cells and platelets which was dependent on the proteolytic activity of the vesicles.

Further study will be required in order to evaluate whether gingipains-R in their various forms activate PARs *in vivo* and determine the subsequent cellular effects. Interestingly, it was recently found that serine peptidases, thrombocytin and PA-BJ,

isolated from the venom of *Bothrops atrox* and *Bothrops jararaca*, respectively, increase intracellular calcium levels in human platelets and induce platelet aggregation and granule secretion (Santos *et al.*, 2000). The effect of these viper venom peptidases on platelets was shown to be mediated by activation of PAR-1 and PAR-4 on platelets (Santos *et al.*, 2000). The bacterial protease streptokinase has also been recently found to activate PAR-1 and induce platelet activation (McRedmond *et al.*, 2000). Thus, the findings presented in this thesis may be part of an emerging insight into how bacterial proteases by acting through PARs can affect the host's cells.

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