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Mentors And The Butterfly Effect: Triggers For Discovering Signalling by Proteinases via Proteinase-Activated Receptors (PARs) And More

Abstract

The essential role of proteinases as regulatory digestive enzymes, recognized since the late 1800s, has been underscored by the discovery that more than 2% of the genome codes for proteinases and their inhibitors. Further, by the early 1970s it was appreciated that in addition to their digestive actions, proteinases can affect cell function: (1) by the generation or degradation of peptide hormones and (2) by the direct regulation of signalling by receptors like the one for insulin. It was the discovery in the 1990s of the novel G-protein-coupled 'proteinase-activated receptor' (PAR) family that has caused a paradigm shift in the understanding of the way that proteinases can regulate cell signalling. This overview provides a perspective for the discovery of the PARs and my laboratory's role in (1) understanding the molecular pharmacology of these fascinating receptors and (2) identifying the potential pathophysiological roles that the PAR family can play in inflammatory disease. In this context, the overview also portrays the essential impact that seemingly minor comments/insights provided by my lifelong mentors have had on kindling my intense interest in proteinase-mediated signalling. The 'butterfly effect' of those comments has led to an unexpectedly large impact on my own research directions. Hopefully my own 'butterfly comments' will also be heard by my trainees and other colleagues with whom I am currently working and will promote future discoveries that will be directly relevant to the treatment of inflammatory disease.

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The 'butterfly effect' and its impact on trainee directions.

The principal focus of this overview will be on the ability of proteinases to trigger hormone-like signals via proteinase-activated receptors (PARs) and other mechanisms. A subtext of the synopsis will deal with an essential process that has shaped the development of my own research programme from its initial steps to the point where the main emphasis in my laboratory is now on proteinase-mediated signal transduction. It is only in retrospect that the critical effect of seemingly casual comments made by my outstanding mentors can be appreciated. Like the classical 'butterfly effect'[1], the impact of my mentors' apparently offhand observations barely made a ripple in my consciousness at the time; but, affecting each stage of the trip to my present situation, those words can be seen to have developed cumulatively into a veritable tsunami that has pushed me to where I am today. My guess is that at the time of their comments (summarized in Table 1), even my mentors had no idea of the importance of their insights in shaping my career at each of its stages (Photos: Figure 1). The message of the subtext: mentorship is key for any research career and the 'butterfly effect' of a mentor's insight and offhand comments can never be underestimated. In this regard, I am honoured to record that early on in my career, it was one of these 'butterfly comments' made by Dr. Henry Friesen to me across his desk in Winnipeg that has contributed to the tsunami I mention above.

The following sections deal mainly with the roles that proteinases play as hormone-like inflammatory mediators. A brief perspective will also be provided to illustrate the multiple influences that played a role in developing my conceptual framework for this topic. On looking back, each stage of earlier work makes sense in terms of the current direction as outlined in Figure 1. At each individual time point, the eventual outcome, my focus on proteinase-mediated signalling, could not have been predicted. Thus, at the end of the road, one recognizes where one is; but, at the beginning, it is never clear as to where one is going. What is clear is that, as emphasized to me by my brushwork mentor, Chin-Shek Lam, 'the first step has to be in the right direction'.

Diverse training experience and an integrated approach to a problem.

To understand the diverse mechanisms whereby proteinases regulate cell function, it is necessary to be familiar with issues ranging from chemical synthesis and peptide structure-activity relationships to clinical aspects of inflammatory disease. By sheer luck, but NOT by design, my training trajectory touched

TABLE 1: Butterfly Comments And Their Ultimate Effectsa

Mentor	Comment	Ultimate Impact
H. D. Gesser, M.Sc. thesis supervisor, U of Manitoba, 1962-64	"I still have something to say; and it would be a shame not say it"	Long-term commitment to a research career; enthusiasm for taking on 'crazy-idea projects'
H. K. F. Blaschko, Doctoral Thesis Advisor with D.B. Hope, Oxford University, 1964-67	"Learn your peptide chemistry first from Derek Hope; then go rowing. Yes, I'll be happy to provide you with a letter of introduction to Professor Talalay."	Neurophysin-oxytocin-vasopressin crystals obtained first; then went up-river two positions in Torpids rowing races. I was well received by Prof. Talalay due to the letter of introduction
P. Talalay, former Department Head, Pharmacology & Experimental Therapeutics, Johns Hopkins School of Medicine; Mentor 1968-present	"Welcome to our Department: let me know how I can help you. By the way, disseminated cancer can be controlled or even prevented."	Long-term mentorship, finding a home in the Hopkins Department of Pharmacology & Therapeutics with substantial support and continued career guidance
P. Cuatrecasas Post-Doctoral supervisor, 1972-73; Mentor, 1972-present	"First Insulin and EGF bind, then something happens. Go figure it out. By the way, trypsin and thrombin have insulin-like effects, just like EGF"	Long-term mentorship, generating an interest in the hormone-like actions of proteinases, ultimately leading to studies of PAR structure and function
D. S. Coffey, former Director, Research Laboratories, Department of Urology The Johns Hopkins Hospital; Mentor, 1969-present	"Welcome to our Department: let me know how I can help you. By the way, PSA is a serine proteinase"	Long-term mentorship, focusing attention on the serine proteinase activity of PSA/KLK family members, leading to the discovery that the KLKs can regulate tissue function via PAR signalling
C.S. Lam, brushwork mentor, 1985-90	"On any journey, ensure that the first step is in the right direction"	Insight about the value of a correct initial direction and mentorship for a brushwork painting second career

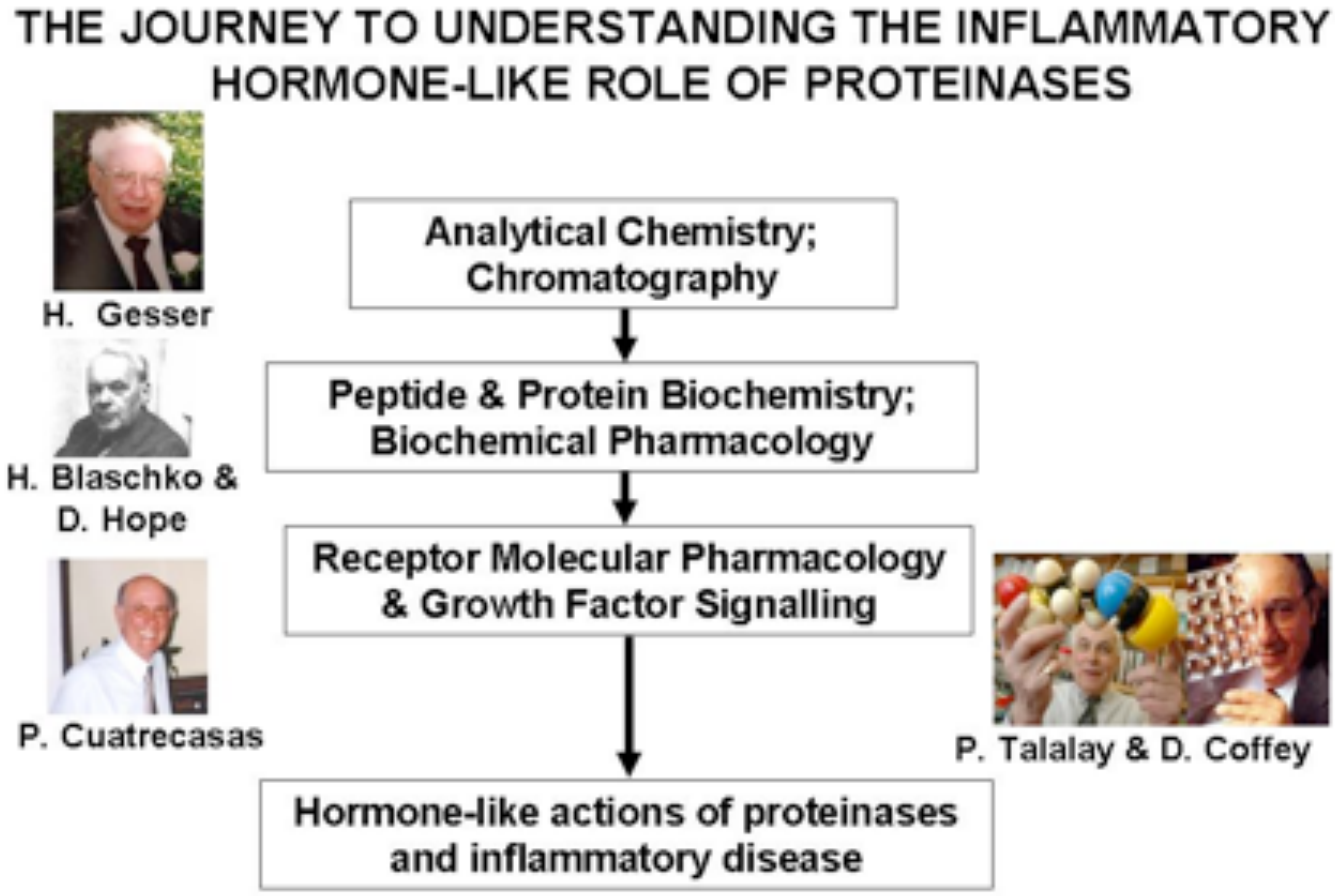

FIGURE 1: Mentors and their impact on concepts of proteinases as inflammatory mediators.
The figure illustrates the skills acquired at each stage of my training that proved to be essential for dealing with the mechanisms that account for inflammatory signalling by proteinases. At each stage, the photographs identify the mentors, who with their support and comments recorded in Table 1, have had an essential influence on the trajectory my research directions have taken. Unfortunately, a photo for Dr. Derek Hope, my doctoral supervisor, was not available to show along with the photo of Dr. H.K.F. Blaschko who was also a mentor in the Oxford Pharmacology Department.

on all of these areas and more. My personal journey began with a crazy-idea-M.Sc. thesis project that today might be coined as a 'search for a biomarker for schizophrenia'. That project, which consolidated my love of crazy idea projects, and which was encouraged by my M.Sc. supervisor, Dr. Gesser, aimed to use pyrolysis-gas chromatography to identify sweat metabolites related to disease[2]. At that point, it seems as though one could not have been farther from isolating proteinases from allergens to test their ability to signal to cells. A recent and ongoing project in my lab, identifying allergen-derived PAR-activating proteinases, uses the same chromatographic principles that I learned during my M.Sc. training. Upon completing my M.Sc., my subsequent doctoral project involved understanding the complementary interactions between peptide hormones (oxytocin and vasopressin) and their purified neurophysin binding proteins. That insight led to their crystallization as stoichiometric complexes[3] and fostered my interest in peptide structure-activity relationships. It was unforeseen that the same principles would eventually be relevant to the study of the structure-activity relationships in peptide-stimulated activation of proteinase-activated receptors, as outlined in sections that follow. The training continued with post-doctoral work

dealing with the anabolic actions of insulin and epidermal growth factor (EGF). It was clear at that time that there were striking parallels between these growth factor signalling pathways and the effects of proteinases like thrombin. That perspective consolidated for me the relationship between the growth factor actions of hormones like insulin and EGF, and the mitogenic effects of proteinases like trypsin and thrombin. Thus, the stage was set for me to explore the signalling mechanisms in common between proteinases and polypeptide growth factors. That theme underlies much of the data to be outlined below. As an integrator for the research expertise, my medical training, including training in internal medicine and an internship treating individuals with diabetes and inflammatory disease, provided the perspective for tackling problems of clinical importance. It is that perspective that has led to the insight that, as mediators of the innate immune response, proteinase-activated receptors (PARs) can play central roles in inflammatory diseases. Thus, the following areas of expertise gained from seemingly unrelated previous training are central to the work currently ongoing in my laboratory: (1) organic and inorganic chemistry and principles of chromatography (gas and liquid) used in my M.Sc. and doctoral work; (2) structure-activity relationships for protein-protein interactions and drug action (doctoral work); and (3) principles of insulin-like anabolic cell responses and measuring hormone-receptor interactions (post-doctoral experience). The following sections provide an overview of hormone-like signalling triggered by proteinases. In retrospect, it can be seen that all of the areas of expertise illustrated in Figure 1 have had a bearing on my understanding of the mechanisms whereby proteinase-mediated signalling affects inflammation.

Proteinases As Hormone-Like Signal Messengers

Proteolytic regulation of cell function.

The importance of proteinases as regulatory digestive enzymes, recognized since the late 1800s[4,5], has been driven home by the discovery that more than 2% of the human genome codes for either proteinases (also colloquially referred to as proteases) or their inhibitors[6,7]. The many mechanisms whereby these enzymes regulate cell function (Figure 2) have only been elucidated over the past 40 years. For instance, the discovery of hypotensive peptide principles in urine that have contractile activity in uterine smooth muscle[8], led to the finding in the late 1960s that proteinases generate inflammatory kinins from their polypeptide precursors (summarized by Erdos[9]). At that time, the mechanisms for the processing of proinsulin to insulin were just being established[10]. Thus, by the late 1960s, the first of the five mechanisms outlined in Figure 2, whereby proteinases could affect cell function, namely the generation or degradation of active polypeptides, was established. Since that time, proteinases have taken on a new life as hormone-like signal messengers. What was not fully appreciated until recently is that proteinases, such as trypsin and chymotrypsin, act to mirror the effects of peptide hormones on tissues. For instance, in the mid-1960s the Riesers found that pepsin and chymotrypsin could reproduce insulin's action to promote glycogen formation in a rat diaphragm preparation[11,12]. Trypsin was subsequently found to mimic the action of insulin in isolated fat cells to stimulate glucose oxidation and inhibit lipolysis[13]. This insulin-like action of trypsin has been shown to result from its cleavage of a negative regulatory domain of the insulin receptor alpha-subunit, so as to trigger insulin signaling[14]. In another experimental model, the serine proteinases thrombin and trypsin were found, like insulin and epidermal growth factor (EGF), to stimulate cultured cell mitogenesis by acting at the cell surface[15-19]. It then took more than a decade to discover the proteolytically activated receptor targets for these actions of thrombin and trypsin.

Proteinase-activated receptors (PARs) as targets for cell signaling.

It was the successful search for the receptor responsible for the platelet-activating and mitogenic functions of thrombin that resulted in a paradigm shift in understanding proteinase-

TABLE 2: PAR-Activating Peptides (PAR-APs)

Receptor	PAR-APs and *standard inactive* control peptides
PAR1	TFLLR-NH_2 *FTLLR-NH_2; RLLFT-NH_2*
PAR2	SLIGRL-NH_2; 2furoyl-LIGRLO-NH_2 *LSIGRL-NH_2; LRGILS-NH_2*
PAR4	AYPGKF-NH_2 *YAPGKF-NH_2*
PAR3	Does not activate via PAR-APs; its tethered ligand sequences (e.g. TFRGAP-NH_2), activate PARs 1 and 2

The table lists for PARs 1, 2 and 4, the receptor-selective PAR-activating peptide sequences along with 'partially scrambled or reverse-sequence (underlined)' receptor-inactive peptides that can be used as 'controls' for stimulating cells and tissues.

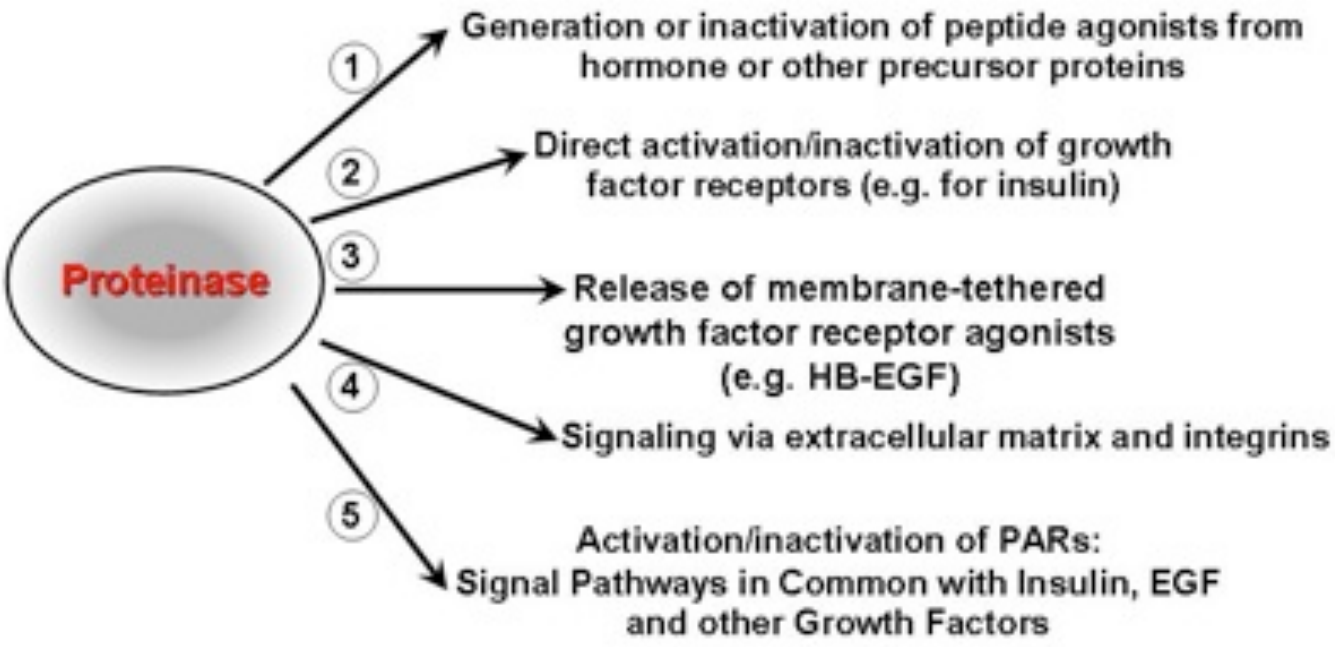

FIGURE 2: Mechanisms whereby proteinases generate hormone-like signals.
The scheme shows five distinct mechanisms, including PAR signalling, that account for the ability of proteinases to affect cell and tissue function.

mediated signaling[20,21]. Not only was a thrombin-responsive G-protein-coupled receptor (GPCR) cloned independently by two laboratories, but a unique mechanism of activation was established[21] that involves the proteolytic unmasking of an N-terminal sequence that functions as a tethered ligand by binding to the receptor extracellular domains to stimulate receptor signaling (Figure 3). This unusual GPCR superfamily member is now designated as a Proteinase-Activated Receptor, given the acronym, PAR. The first receptor of this kind discovered, responding to thrombin, is termed PAR1. Remarkably, synthetic peptides modeled on the revealed N-terminal tethered ligand sequence are able, on their own, to activate receptor signaling in the absence of proteolysis[21]. Thus, the peptide, SFLLRN, representing a PAR1 tethered ligand sequence, is able to activate thrombin receptor/PAR1 signaling and to cause human platelet aggregation, mimicking thrombin action.

Discovering multiple PARs representing a novel GPCR family.

At the same time as PAR1 was cloned, we had established that thrombin had signal transduction pathways in common with EGF to cause smooth muscle contraction via a non-receptor tyrosine kinase signal[22]; and we realized further that the PAR1-activating peptide, SFLLRN, did not mimic the ability of thrombin to activate rodent platelets[23]. We therefore proposed: (1) that PAR1, like EGF, could trigger rapid tissue responses via Src-related non-receptor tyrosine kinase signal pathways and (2) that PAR1, very likely, had closely related receptor subtypes, akin to the alpha- and beta-adrenoceptors identified some time ago using the strategy developed by Ahlquist[24]. We, therefore, used classical smooth muscle bioassays, which involve tyrosine kinase signal pathways, to establish structure-activity relationships for a series of PAR1-activating peptides. The data clearly demonstrated the presence of PAR subtypes[25,26]; but despite our efforts, we were not successful, at that time, in cloning other members of the PAR family. Coincidentally, a fortuitous isolation of a bovine substance K-related receptor clone from a mouse genomic library resulted in the identification of the second member of the PAR family, PAR2[27]. Based on the sequence of the proposed PAR2 tethered ligand (SLIGRL), we realized, from our own structure-activity data, that a tethered ligand peptide with a non-aromatic residue (leucine) at the second position instead of phenylalanine would not activate PAR1, and we rapidly established the ability of the PAR2-activating peptide, SLIGRL-NH2, to regulate endothelium-dependent vasorelaxation in a

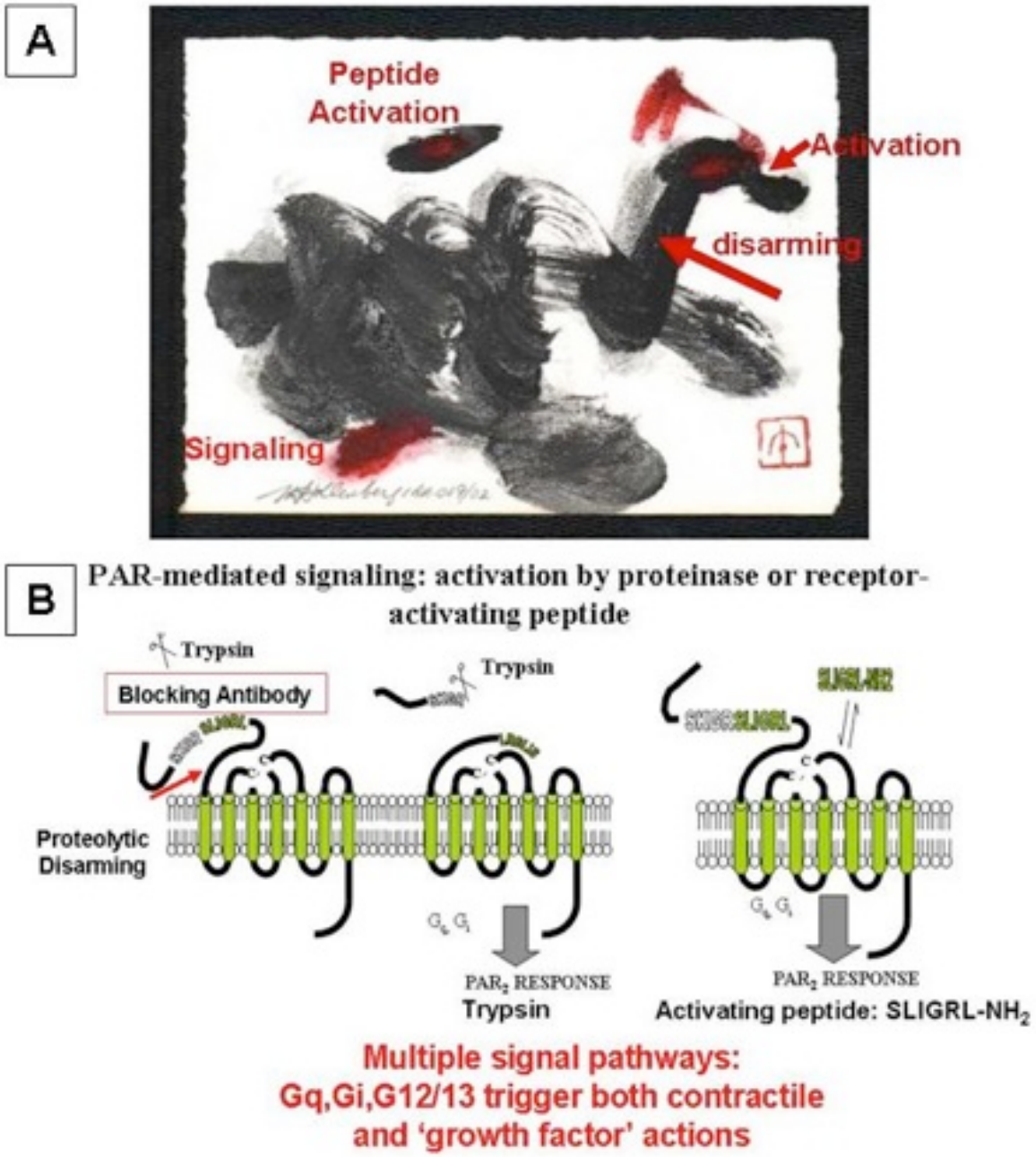

FIGURE 3: Proteinase-activated receptor signalling mechanisms.
A. The brushwork image (upper figure), illustrates the dynamic membrane-localized mobility of a PAR as it triggers a signal (bottom, red) either by the unmasking of a 'tethered ligand' domain (red-centred oval sequence, upper right) or by the action of a synthetic tethered-ligand-derived PAR-activating peptide (red-centred oval peptide, upper, middle). The ability of a proteinase antagonist to silence the receptor by disarming the tethered ligand sequence (right, middle) is also shown. B. A conventional 7-transmembrane receptor PAR2 cartoon (left panel) shows the activation of rat PAR2: (1) by trypsin-mediated exposure of its tethered ligand (middle panel) and (2) by a synthetic peptide, SLIGRL-NH_2 that mimics the tethered ligand (right panel). The figure also shows (left panel): (1) silencing of PAR2 by proteolytic disarming of the tethered ligand sequence and (2) blocking trypsin activation of PAR2 by preventing access of trypsin to the activation/cleavage site with a cleavage-site-targeted blocking antibody. In keeping with the mobile-floating receptor paradigm of hormone action[50,51], PAR2 is shown (bottom, Fig. 3B) to interact independently with several G-proteins like Gq, Gi and G[12-13], depending on the stimulating agonist. Thus, PAR2 is capable of biased signalling caused by either peptide or enzyme activation[56,84].

rat aorta preparation[28]. Thus, unexpectedly, the structure-activity approach learned in the context of the vasopressin/oxytocin-neurophysin project of a doctoral thesis, where the principles outlined by Ahlquist[24] were consolidated, served as the basis for the validation of the presence of functional thrombin-PAR receptor subtypes in vascular tissue[29] and to lead to our cloning of rat PAR2[30]. By this time, PAR2 from mouse, rat and human[31,32] sources had been cloned and the cloning of the last two members of the PAR family (PARs 3 and 4) followed shortly thereafter[33-35]. Although members of the PAR family (PARs 1, 3 and 4) are seen clearly to play a role in thrombin-mediated platelet and endothelial cell function, the question remains: what do the PARs, PAR2 in particular, do in other tissues?

PAR-activating peptides as probes for PAR function.

Three approaches have been used to assess the potential physiological functions of the PARs: (1) evaluation of the effects of receptor-selective PAR-activating peptides, (2) studies with PAR knockout mice and (3) use of PAR antagonists. Of the three approaches, the use of PAR-activating peptides (PAR-APs, Table 2) has provided by far the most insight, in conjunction with the use of PAR-null mice. Unfortunately, attempts to develop clinically-effective PAR antagonists has been disappointing, except for PAR1 (see review[36]). Based on our own structure-activity data and information in the literature, we designed a set of receptor-selective PAR-activating peptides (PAR-APs), along with scrambled sequence control peptides that cannot activate PARs (Table 2). The key to the successful use of these agonists has been: (1) to perform concentration effect curves for one or more peptide agonist and (2) to use more than one PAR-inactive control peptide. Thus, even though some of the PAR-activating peptides can have off-target effects[37-39], the use of PAR-AP structure-activity information in the context of studies with PAR null mice can validate a role for PARs 1, 2 and 4 in any given system[40-42].

Inflammatory and vascular actions of PARs.

Based on our vascular and gastric smooth muscle bioassay systems used to evaluate the pharmacological actions of PAR-activating peptides, we were able to establish a functional role for PARs 1 and 2 in intact smooth muscle tissues[28,40]. It was with our rat PAR2-targeted antibody that the more widespread presence of this receptor was localized in intestinal tissue on a variety of cell elements including epithelial, smooth muscle, vascular and neuronal elements[43]. From that study it was evident that the PARs could affect not only platelets and blood vessels, as was expected from the presence of functional PARs on these tissues, but could also regulate a wide spectrum of tissues, including neuronal targets.

Given the presence of PARs in the gut and other peripheral tissues, and given that inflammatory reactions in the gut had been linked to the secretion of mast cell proteinases into the tissue and circulation[44], we hypothesised that mast cell-released proteinases might cause tissue inflammation by activating PARs. This idea was taken on by a gifted investigator and long-time collaborator, Nathalie Vergnolle, working at the time jointly in my lab and that of my close colleague, John Wallace. The idea was to isolate rat mast cell proteinases and monitor their inflammatory effects via the PARs; however, the literature-based procedures for the chromatographic purification of rat mast cell proteinases did not meet the standards I had come to expect from my doctoral work[3]. Thus, our strategy switched from testing the inflammatory actions of the mast cell enzymes directly, to testing the potential inflammatory actions of the PAR1/2-activating peptides themselves. The results were dramatic (Figure 4)[45,46]. By a selective non-proteolytic activation of PARs 1 and 2 in peripheral tissue using the PAR-APs, it was possible to recapitulate most of the hallmarks of the inflammatory response, including increased blood flow, swelling and pain. It was at that point that my laboratory direction made an about face to focus on the signalling properties of proteolytic enzymes and the roles of PARs and other proteinase targets in inflammatory diseases. What soon became evident was that the PAR activation process leading to inflammation and pain involved a neurogenic mechanism with proteinase targets on both neuronal and non-neuronal tissue elements[47,48]. It became essential to study the molecular pharmacology of PAR signalling in more depth.

Biased PAR signalling can selectively trigger unique tissue responses.

Discussions ongoing in the Cuatrecasas lab in the early 1970s dealt with the mechanisms by which (1) multiple hormones, such as glucagon and ACTH, could affect a common adenylyl cyclase or (2) insulin could trigger such diverse responses in tissues, ranging from anti-lipolysis to anabolic cell biosynthesis. This kind of discussion generated the mobile or floating receptor paradigm of hormone action[49-51] that proposed that (1) multiple receptors could regulate a single cellular effector (e.g., adenylyl cyclase) and (2) an individual receptor, like the one for insulin, could regulate multiple independent cell effectors. Clearly, this paradigm applies to a receptor such as PAR1 that can trigger increases in cellular calcium by activating Gq, whilst decreasing cellular cyclic AMP by interacting with Gi. What is

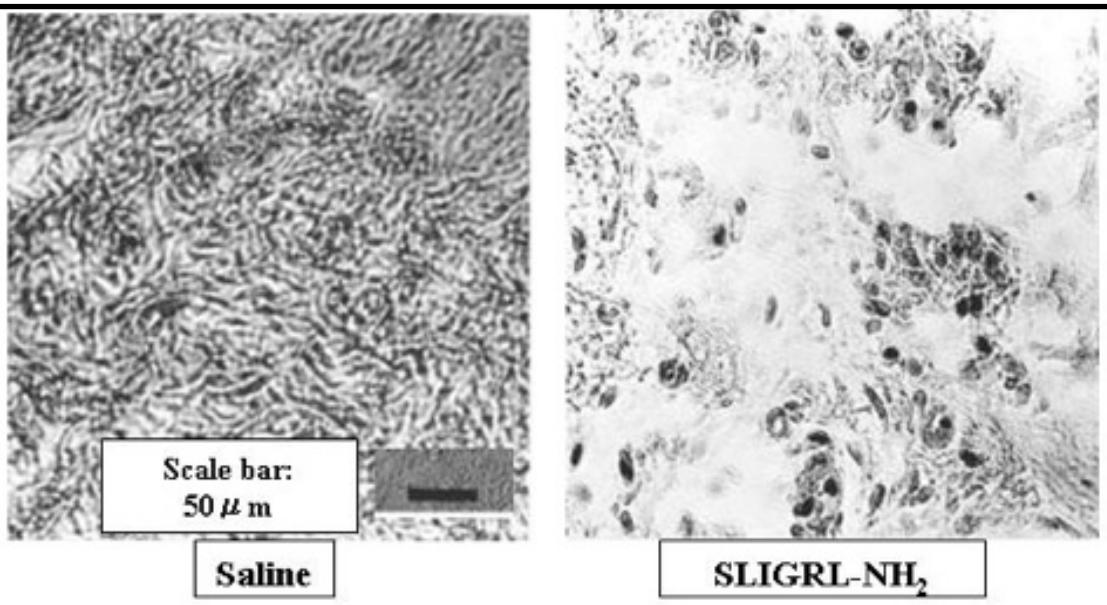

FIGURE 4: Inflammatory action of PAR2 activation in the rat paw.
The histology shows fixed and stained sections of rat hindpaws, 6 h after the intraplantar injection of (1) isotonic saline solution (control, left panel) and (2) a PAR2-activating peptide (SLIGRL-NH_2, 500 μg: right panel). The oedema resulting from PAR2 activation (right panel) caused fluid-filled spaces between the cells and the infiltration of neutrophils (cells with prominent nuclear staining). A control, reverse-sequence peptide (LRGILS-NH_2) did not cause inflammation, resulting in a normal-looking tissue picture the same as on the left (not shown). Scale bar in left panel: 50 μm. Adapted from reference 45.

now appreciated is that this process can lead to biased signalling, whereby the activation (or inhibition) of a given receptor can affect a unique signal pathway, to the exclusion of other pathways[52]. Work by others has shown that activation of PAR1 by thrombin, matrix metalloproteinases or activated protein-C can result in quite distinct effects, including both inflammatory and anti-inflammatory responses[53-55]. Our own work has shown that this kind of biased PAR signaling can be triggered for PAR2, both by signal-selective PAR-activating peptides and by different inflammation-related enzymes such as neutrophil elastase[56]. Biased PAR signaling can depend not only on a selective interaction of a PAR with a targeted G-protein (e.g., Gi) but also on a beta-arrestin recruitment mechanism[53,57]. From my own perspective, the discovery of biased signalling by PARs represents a natural evolution of the mobile receptor hypothesis coined in the mid 1970s, which can now be seen as having tremendous therapeutic potential in terms of developing signal-biased drugs for the PARs and other GPCRs[52]. To understand the impact of proteinase-triggered signalling on inflammation fully, it is therefore necessary to understand the mechanisms of biased PAR signalling.

Stimulating PAR and non-PAR targets for PAR-activating peptide and proteinase-mediated signalling.

Having established the PARs as prime targets for proteinase-mediated signalling, it is important to re-emphasize that both the proteinases and the PAR-activating peptides can signal via non-PAR mechanisms. As outlined above and illustrated in Figure 2, mechanisms other than those involving the PARs can account for proteinase-triggered signals; and the PAR-activating peptides can stimulate PAR-independent signalling via other targets, as we[37,38] and others have found[39]. As a consequence, in a given setting, the actions of proteinases or PAR-agonists may be due both to PAR-activated and PAR-independent mechanisms, as we have found for adipocyte-triggered vascular regulation[38] and as we observed for the regulation of intestinal transport[58]. To sort out the PAR from non-PAR targets of either PAR-activating peptides or enzymes, it is essential, as outlined above, to use a peptide structure-activity approach for the PAR-APs and to employ PAR-null cells, isolated tissues or intact mice as enzyme and PAR2-AP targets[38].

If PARs are the targets, which are the activating enzymes?

Coagulation proteinases versus other enzymes involved in inflammation.

Unlike other hormone-regulated receptors, the PARs have no classical circulating or locally-produced direct agonists, such as angiotensin or adrenaline. In the case of the PARs, the term 'agonist' must be re-interpreted. In a sense, although the direct PAR-regulating agonist is the unmasked tethered ligand, the proteinases themselves must be considered as the physiological PAR regulators; and there can thus be multiple proteinase agonists that can activate the PARs as well as multiple proteinase antagonists that by removing the tethered ligand sequence (disarming: Fig. 3A, right-middle; Fig. 3B, upper left panel), can silence PAR signalling. Well-appreciated are the roles of the coagulation cascade serine proteinases as endogenous PAR-regulatory enzymes. Thrombin, Factor VIIa/Xa, plasmin and activated protein-C can all be seen as physiological agonists of PARs 1, 2 and 4. The likely role of PAR3 as a co-factor for targeting PARs 1 and 4 by thrombin is also accepted, although direct autonomous signalling via PAR3 by enzymes other than thrombin activation[59] has not is been evaluated in any depth. For PAR2, which is not activated by thrombin, but is triggered by the Tissue Factor-Factor-VIIa/Xa complex, trypsin has been suggested as an endogenous activator, since trypsin readily activates PAR2 signalling[27]. Further, other members of the trypsin family, which can be expressed in tissues apart from the pancreas, such as trypsin IV, can activate both PAR2 and PAR4, [60,61]. Thus, the proteinases found to activate the PARs shortly after they were cloned (thrombin, other coagulation proteinases and trypsin) can be thought of as physiological regulators *in vivo*. It is becoming evident that a much wider array of endogenous proteinases can now be considered as physiological regulators of PAR function, not to mention the pathogen-derived proteinases like pseudomonas elastase that can also regulate PARs in pathological settings[62]. The ability of a variety of proteinases to trigger biased signalling by the PARs, without unmasking a classical tethered ligand sequence[56] adds another layer to this issue of identifying physiological regulators of the PARs.

Metalloproteinases (MMPs), kallikrein-related peptidases (KLKs) and neutrophil proteinases as endogenous PAR regulators. Metalloproteinases (MMPs).

The first indication that PARs could be activated by proteinases other than trypsins or proteinases of the coagulation cascade came from the discovery that matrix metalloproteinase-1 (MMP-1) could stimulate the invasion of breast carcinoma cells in a xenograft model by activating PAR1[63]. Surprisingly, MMP-1 was found to activate PAR1 by cleaving at a non-canonical site to unmask an unusual tethered ligand (PRSFLLR) that causes biased PAR1 signalling[64], as appears to be the case for MMP-13[65]. The impact of MMPs on signalling by the other PARs has not yet been thoroughly evaluated. Thus, paracrine signalling in tissues via a MMP-PAR1 activation mechanism appears to represent a physiological way by which MMPs can regulate tissue function[55].

Kallikrein-related peptidases (KLKs)

When first cloned, human PAR2 was found to be quite highly expressed in prostate tissue[31,32], long recognized as a source of the serine proteinase family of kallikrein-related peptidases (KLKs[66]: formerly termed 'tissue kallikreins'). KLKs such as prostate-specific antigen-KLK3 have proved of intense interest as prostate cancer biomarkers, in keeping with the concepts I became familiar with during my M.Sc. work in the Gesser lab, trying to identify a schizophrenia biomarker[2]. Despite their role as biomarkers, the tissue function of KLKs is, as yet, largely unknown. It was a valued mentor of mine at Johns Hopkins, Don Coffey, who, with Bill Isaacs, pointed out over 30 years ago that the canine equivalent of PSA is a trypsin-related serine proteinase[67]. Based on this information remembered from my M.D. training days, I hypothesised that the KLK family of serine proteinases might act in a paracrine manner to regulate tissue function by activating PARs. That hypothesis, driving the research of a talented doctoral trainee, Dr. Katerina Oikonomopoulou in the Diamandis laboratory, in collaboration with my own lab, has been amply supported by our own and others' findings, that a number of the KLKs can indeed regulate PAR function by either activation or inactivation/disarming[68-70]. Of the KLKs we have evaluated, KLKs 5, 6, 8 and 14 each have a differential ability to regulate KLK function. Further, our work has shown that KLK14 on its own can cause an inflammatory response in the paw[71], equivalent to that shown in Figure 4. Of importance, in the skin disorder termed Netherton syndrome, a genetic defect in a serine proteinase inhibitor (SPINK5) that would inhibit skin KLK14 action, it is thought that unchecked KLK proteinase activity plays an important role[72]. Further, a dysregulation of PAR2 appears to be involved in a murine model of Netherton syndrome[73]. Thus, it is probable that KLKs in concert with PAR signalling will be found to be important factors in the pathophysiology of disorders of the skin and, possibly, the prostate.

Neutrophil proteinases.

In the setting of acute inflammation, neutrophil influx represents one of the first indices of tissue damage. The secretion of neutrophil proteinases has a dramatic impact on tissue function. Our own work has found that neutrophil elastase can both disarm PAR2 for activation by trypsin and activate PAR2 in a biased way to stimulate MAPKinase but not calcium signalling[56]. Similarly, neutrophil proteinases can disarm PAR1 for thrombin signalling and can either activate or disarm PAR4[74-76]. Thus, it is possible that one physiological aspect of neutrophil proteinase function involves a disarming of PAR1 and an activation of PAR4, combined with a trypsin-disarming/biased signalling effect via PAR2. All of these effects due to PAR regulation would be expected to have an impact on the inflammatory response involving neutrophils. Other proteinases involved in the innate immune response, like the complement system proteinases, one of which (MASP-1) can target PAR4[77], may also be found to be physiological regulators of PAR function. Clearly, the search for the in vivo regulators of PAR function is just in its infancy.

PARs: Unexpected Keys to Inflammatory Disease

An approach to defining PAR pathophysiology: Tissue bioassays and disease models.

A key issue to deal with is this: once PARs are activated *in vivo*, what is their function? In addition to the use of cell culture and receptor expression studies to understand PAR molecular pharmacology (e.g., see reviews[78,79] for a summary), we have employed a number of organ bath-based tissue assays *and in vivo* disease models to evaluate the potential role(s) that different PARs may play in a variety of physiological settings (see Section 5, reference 79). The use of the rodent hindpaw edema-inflammation model, along with behavioural monitoring of pain, has already been described above to illustrate the potential role of the PARs in peripheral inflammatory disease[45,46]. Through the use of appropriate animal models, where feasible involving the use of PAR null mice, it has proved possible to establish a proof of concept that PARs 1 and 2 and 4 can play roles in a variety of inflammatory diseases ranging from platelet and vascular dysfunction to arthritis, colitis, asthma and inflammation-neurodegeneration of the central nervous system[78,79]. It was not at all anticipated that PARs would represent novel keys for understanding proteinase functions in the setting of inflammation. Of note, quite apart from its expected role in vascular disease, PAR1, along with PAR2, has been found to be integrally involved in the process of epithelial tumour cell growth and invasion[80]. Finally, pathogen-derived proteinases like those present in insect allergens can also promote inflammation by activating PARs[81]. Thus, in many pathological settings, the PARs appear as attractive therapeutic targets.

Will targeting PARs cure inflammatory disease and cancer?

As summarized in the previous paragraph, proof of principle using PAR null mice and other approaches has pointed to roles for PARs as a common denominator in a variety of inflammatory settings including cancer metastasis. Inspired by the conviction expressed by my long-time mentor, Dr. Paul Talalay, that even disseminated cancer can be controlled or prevented (Table 1), my question is as follows: will PAR antagonists (or agonists) be of value in humans to treat cancer or other inflammatory disease? Indeed, in model systems, blocking PAR action has succeeded in treating arthritis, colitis and asthma, ***if you are a mouse***[81-83]. In human trials, however, the clinical use of PAR-targeted antagonists has so far met with limited success, for a number of reasons discussed at more length elsewhere[36]. The issues that have so far impeded the development of clinically useful PAR antagonists relate to (1) the unexpected challenge of designing systemically useful PAR2-targeted agents, in contrast with the success of developing PAR1 antagonists for use *in vivo* in humans and (2) the issue of synthesizing compounds with appropriate pharmacokinetic properties for use in humans. An additional unappreciated aspect of PAR signalling, that has yet to be dealt with by PAR antagonists developed to date, relates to biased PAR signalling by proteinases. PAR activation that occurs via the proteolytic unmasking of a novel non-canonical tethered ligand sequence may prove refractory to inhibition by agents that target only the 'canonical' tethered ligand. This issue of biased enzymatic signalling via the PARs as it may relate to developing signal-selective antagonists remains to be explored in depth. That said, there is indeed cause for cautious optimism, since the therapeutic use of PAR-targeted drugs to date has involved only the cardiovascular system, in attempts to mitigate platelet-triggered pathologies. Other areas in which blocking PAR1 function may be of clinical value have yet to be investigated in depth. The use of PAR antagonists to treat colitis, tumour invasion, pain, itch and other skin pathologies, shows considerable promise, particularly since some of these conditions may benefit from the localized delivery (e.g., skin and gut) of PAR-targeted therapeutic agents. The dramatic roles that PAR1 and PAR2 play in tumour cell growth and metastasis, as discussed above, also point to a key area in which PAR or thrombin antagonists may prove of value.

Summary and a Look To The Future

What is evident from the route I have taken to understand the inflammatory hormone-like roles played by proteinases and their PAR signalling targets (Figure 1) is that at each stage there has been a mentor who imparted a subtle impact that, like the butterfly effect, was magnified greatly over time. The implication of this effect, which has also been evident in the careers of many successful investigators in the past, is that as supervisors-mentors of our own trainees, we are all 'butterflies' responsible for imparting small changes in our generation that then have large impacts on the next. Even though the effects of each interaction we have with trainees (and colleagues) may be small or even barely noticed at the time, the ultimate impact can indeed be substantial. Thus, exchanges even between only a few individuals in a restricted group at one time point can ultimately have a major effect on the entire population at a later date. A few wise words whispered in receptive ears at one time can be amplified dramatically in the future.

The work that has had its origins in my thinking about biomarkers and peptide structure-activity relationships for oxytocin/vasopressin-neurophysin interactions[2,3] and that has been amplified by considering the anabolic actions of thrombin, has ultimately led to me and my collaborators playing a significant role in (1) furthering the understanding of the hormone-like roles played by proteinases and (2) uncovering the unexpected inflammatory actions and molecular pharmacology of the PARs. The dream is that the insights about the mechanisms and roles that proteinases and their PAR targets can play in inflammatory settings will ultimately lead to therapeutic modalities that can mitigate the impact of diseases like colitis arthritis asthma and cancer. It is with that exciting possibility in mind that I look forward to future work in my laboratory.

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List of Abbreviations

APC	Activated protein-C
EGF	Epidermal growth factor
GPCR	G-protein-coupled receptor
PAR	Proteinase-activated receptor
PAR-AP	PAR-activating peptide

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