BJP BRITISH JOURNAL OF PHARMACOLOGY

BPS BRITISH PHARMACOLOGICAL SOCIETY

British Journal of Pharmacology (2009), 158, 936–946

0007-1188/09
www.brjpharmacol.org

THEMED SECTION: MEDIATORS AND RECEPTORS IN THE RESOLUTION OF INFLAMMATION

REVIEW

Exploiting the Annexin A1 pathway for the development of novel anti-inflammatory therapeutics

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The appreciation that the inflammatory reaction does not 'spontaneously' finish, but rather that inflammatory resolution is an active phenomenon brought about by endogenous anti-inflammatory agonists opens multiple opportunities for a reassessment of the complexity of inflammation and its main mediators. This review dwells on one of these pathways, the one centred around the glucocorticoid-regulated protein Annexin A1 and its G protein-coupled receptor. In recent years, much of the knowledge detailing the processes by which Annexin A1 expresses its anti-inflammatory role on innate immunity has been produced. Moreover, the generation of the Annexin A1 null mouse colony has provided important proof-of-concept experiments demonstrating the inhibitory properties of this mediator in the context of inflammatory and/or tissue-injury models. Therefore, Annexin A1 acts as a pivotal homeostatic mediator, where if absent, inflammation would overshoot and be prolonged. This new understanding scientific information could guide us onto the exploitation of the biological properties of Annexin A1 and its receptor to instigate novel drug discovery programmes for anti-inflammatory therapeutics. This line of research relies on the assumption that anti-inflammatory drugs designed upon endogenous anti-inflammatory mediators would be burdened by a lower degree of secondary effects as these agonists would be mimicking specific pathways activated in our body for safe disposal of inflammation. We believe that the next few years will produce examples of such new drugs and the validity of this speculation could then be assessed.
British Journal of Pharmacology (2009) **158,** 936–946; doi:10.1111/j.1476-5381.2009.00483.x

This article is part of a themed issue on Mediators and Receptors in the Resolution of Inflammation. To view this issue visit http://www3.interscience.wiley.com/journal/121548564/issueyear?year=2009

Keywords: anti-inflammation; neutrophils; migration; trafficking; intravital microscopy; GPCR; induction; drug discovery; peptides

Abbreviations: FPR, human formyl peptide receptor; Fpr1, mouse formyl peptide receptor; Fpr-rs, mouse formyl peptide receptor-related sequence; FPRL-1, human FPR-like 1; GPCR, G protein-coupled receptor

Introduction

The awareness of the complexity of the experimental inflammatory reaction has steadily increased in the past decade, in concomitance with the discovery of endogenous inflammatory and pro-resolving pathways (Nathan, 2002). Together with a few other laboratories worldwide, we have pioneered the concept that the inflammatory response follows – in an ideal fashion – a bell-shaped curve such that an acute strong inflammatory phase is followed by a pro-resolving phase with dampening of the response (Perretti, 1997; Serhan and Savill, 2005; Serhan *et al.*, 2007). This sequence attained by an active involvement of inhibitory pathways and mediators (Gilroy *et al.*, 2004; Serhan *et al.*, 2007). Figure 1 illustrates the necessary balance between pro-inflammation and anti-inflammation in order to assure homeostasis is restored in the inflamed tissue after proper resolution. Furthermore, this balance is finely tuned so that alterations, either by an excess of pro-inflammatory mediator expression and/or function or by an augmentation of the endogenous anti-inflammatory arm, would lead to disease (Figure 1).

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Received 13 January 2009; revised 5 August 2009; accepted 11 August 2009

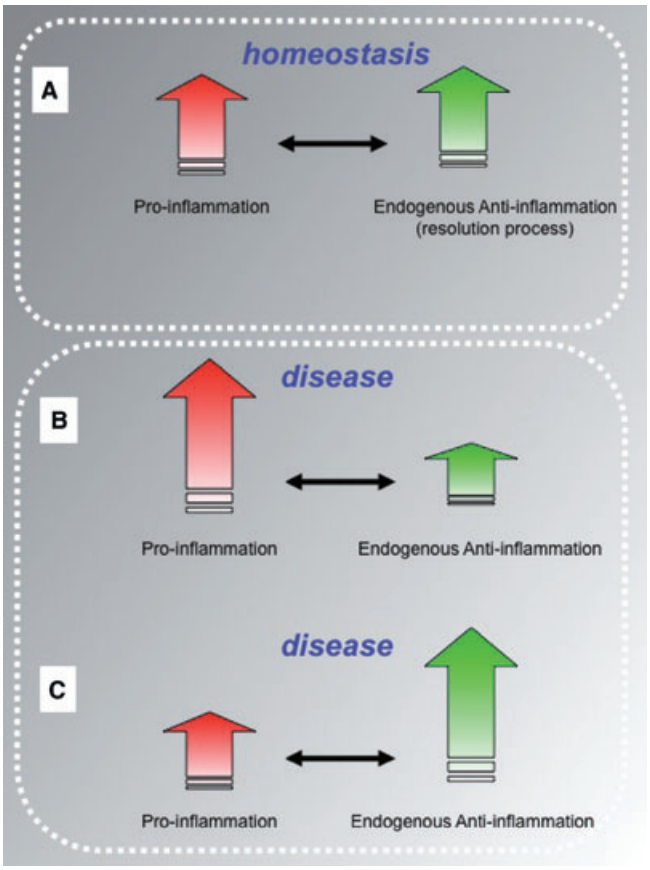

Figure 1 Schematic representation of the required balance between pro- and anti-inflammatory mediators and pathways. (A) An equilibrium between pro-inflammatory mediators and effectors of anti-inflammation is required to assure a prompt yet spatially and temporally restricted inflammatory reaction. This is required for a *proper function* of the inflammatory response the host mounts upon infection and encounter with xenobiotics, so that upon killing and/or disposal of the inflammogen, resolution of inflammation is completed with restoring of tissue physiology and homeostasis regain. (B and C) Pro-inflammation in association with a deficient endogenous anti-inflammatory response, hence with inadequate resolution phase, would lead to disease; the same holds true if an inappropriate pro-inflammatory is produced, so that resolution of inflammation would prevail (e.g. leukocyte adhesion deficiency, whereby lack of leukocyte trafficking is at the basis of patients' inability to fight infections, leading to a poor life expectancy). Acceptance of this important yin/yan in inflammation ensues that chronic inflammatory disease (various forms of arthritis, vasculitis, psoriasis but also perhaps atherosclerosis and reperfusion injury conditions) could be due, at least partly, to an insufficient activation of the endogenous anti-inflammatory response so that resolution pathways are not properly activated and/or operating. Along this line, potentiation of one or more endogenous pro-resolving pathways could be another approach to the therapeutic control of inflammatory diseases.

This review focuses on one of these mediators, the glucocorticoid-modulated protein Annexin A1. Recent reviews have covered multiple aspects of the biology of this protein spanning from the central nervous system (John *et al.*, 2004; Solito *et al.*, 2008) to action upon the adaptive immune system (Gerke and Moss, 2002; D'Acquisto *et al.*, 2008b; Perretti and D'Acquisto, 2009). Here we summarize the actions of Annexin A1 in innate immunity, emphasizing the opportunity that a better appreciation of the mechanisms activated by this protein to inhibit the inflammatory reaction would represent for innovative drug discovery. In fact, as proposed before (Perretti, 1997), we hypothesize that targets activated by endogenous anti-inflammatory mediators could be exploited for novel drug discovery programmes; new molecules obtained and developed in this manner would likely be devoid of major side effects, as their application would be mimicking the way our body naturally disposes of inflammation. Development of adenosine or somatostatin analogues would fit with this approach (Perretti, 1997).

Annexin A1: generalities

Annexin A1 is a 37 kDa protein formed by 346 amino acids. It is the first member of 13 member of a protein family 'The Annexins', grouped together in view of their structural characteristics, including the presence of shared sequences for calcium binding (Gerke and Moss, 2002; Gerke *et al.*, 2005). All annexins consist of a core, which is constituted by four repeats of 60–70 amino acids each, attached to a unique N-terminal region. The core represents the large majority (≥80%) of the protein, whereas the N-terminus likely confers specificity of action to each member of the annexin superfamily of proteins (Gerke *et al.*, 2005).

An important feature of Annexin A1, also shared by other members of the family, is its ability to alter its conformation upon binding to calcium cations (Rosengarth *et al.*, 2001a,b; Gerke *et al.*, 2005). In the presence of a calcium concentration ≥1 mM (e.g. as in plasma or other biological fluids), Annexin A1 undergoes a conformational restructuring allowing phospholipid binding, in particular binding to acidic phospholipids (Rosengarth *et al.*, 2001a,b). Interaction with phospholipids via the core region, sustained by its calcium-binding motifs, is concomitant with a conformational rearrangement of the N-terminal region such that its amino acids are now exposed to the extracellular environment (Rosengarth *et al.*, 2001a). These structural changes are likely to impact on the biology of the protein and, in particular on its ability to interact with potential receptors (discussed below).

Our major interest, in line with the functions of Annexin A1 in the context of inflammation, lies in the role that this protein plays once in the extracellular fluids, including plasma and inflammatory exudates. However we should not overlook the notion that Annexin A1 might be endowed with specific *intracellular roles*. These intracellular properties are also governed by its ability to interact with membranes and possibly again being regulated by the levels of cations in a specific subcellular compartment (Gerke *et al.*, 2005).

An important aspect in the biology of Annexin A1, which will be only superficially covered in this review, is the modulation of protein expression as well as cellular localization by glucocorticoids (Mulla *et al.*, 2005). Indeed, historically Annexin A1 was identified as a glucocorticoid-regulated protein, as earlier observations showed that this class of drugs would augment its levels both in macrophages, lung tissue and kidney mesangial cells (Flower and Blackwell, 1979; Blackwell *et al.*, 1980; Flower, 1985; 1988). It is now evident that the association between glucocorticoids and Annexin A1 is more complex than initially observed.

Dexamethasone and other steroids can rapidly increase cell surface localization of Annexin A1 (Croxtall *et al.*, 1998; 2000Solito *et al.*, 2006); this process does not require *de novo* protein synthesis and is associated with rapid changes in the cellular localization of the protein. More delayed augmentation of cell surface expression of Annexin A1 is consequent to gene activation. Actinomycin D and cycloheximide could inhibit delayed Annexin A1 expression even though the Annexin A1 promotor region lacks a canonical glucocorticoid response element in the Annexin A1 promoter region (Solito *et al.*, 1998a,b). This observation does not exclude the possibility that such regions might be present upstream of the DNA sequences analysed so far, nonetheless it poses the problem of how glucocorticoids could increase Annexin A1 gene expression. In monocytic cells, involvement of specific transcription factors such as nuclear factor interleukin (IL)-6 has been invoked in regulating Annexin A1 gene expression (Solito *et al.*, 1998b). This issue requires further investigation including the validation of such a mechanism in other cell types. Glucocorticouds may also down-regulate Annexin A1 gene expression under some conditions as recently demonstrated in T cells (D'Acquisto *et al.*, 2008a). The role of Annexin A1 in the adaptive although immune response is not covered in the present review (see Perretti and D'Acquisto, 2009 for a recent review) and will not be described here.

Cell sources of Annexin A1

Annexin A1 is widely distributed in the body being found in white blood cells, stromal cells and biological fluids. In the circulation, granulocytes and monocytes are the largest cell source of Annexin A1, with the neutrophils being the predominant cell source in the granulocyte subgroup (Perretti *et al.*, 1996b; Oliani *et al.*, 2002). Lymphocytes have modest expression of Annexin A1, with T cells being positive for the protein (although with nearly a 1:100 the expression observed in neutrophils), whereas B cells (Morand *et al.*, 1995; Mulla *et al.*, 2005; D'Acquisto *et al.*, 2007; Perretti *et al.*, 2009) and platelets are negative for Annexin A1 expression.

In general terms, cell differentiation is associated with a higher expression of Annexin A1 (Kamal *et al.*, 2005; Babbin *et al.*, 2006) and this is evident when macrophages are compared with monocytes prepared from the same donor (Peers *et al.*, 1993); mast cells also express Annexin A1 (Oliani *et al.*, 2000; 2008; Sena *et al.*, 2006; Silistino-Souza *et al.*, 2007) and the same applies to stromal cells such as the fibroblast (Errasfa *et al.*, 1985; Goulding *et al.*, 1996a; Solito *et al.*, 1998a; Dasuri *et al.*, 2004; Tagoe *et al.*, 2008). The latter cell type has been one of the first to be used for the identification of the biological function of the protein. Epithelial cells are also a major source of the protein in compartments such as the lung, the gut and the kidney (Vervoordeldonk *et al.*, 1994; Solito *et al.*, 1998a; Babbin *et al.*, 2006). Kidney mesangial cells are also strongly positive for the protein. Therefore, in simple terms, fully differentiated cell types such as those found in tissues are strongly positive for Annexin A1. Furthermore, cell differentiation and in some cases also cell activation is a major stimulus for Annexin A1 synthesis and up-regulation, although the molecular processes behind this response have not been fully addressed. Examples of this important regulation of the protein expression are seen with tumour necrosis factor-stimulated fibroblasts (Tagoe *et al.*, 2008) or responses that have been reported in epithelial cells (Croxtall and Flower, 1992).

Finally an unsolved mystery in the assessment of Annexin A1 biology is the mode of secretion of the protein. As stated above, we propose that in order to exert its actions and impact onto the inflammatory process, Annexin A1 must be externalized by its cellular sources. However the protein lacks a signal peptide and therefore cannot be secreted via a classical pathway (Muesch *et al.*, 1990; Christmas *et al.*, 1991). We have already mentioned that glucocorticoids can provoke rapid mobilization and externalization of the protein, possibly consequent to rapid phosphorylation by protein kinase C activation (Solito *et al.*, 2006); however, other mechanisms might also exist. In the context of neutrophil biology it was observed that a large proportion of Annexin A1 was contained in subcellular granules and that this pool was externalized upon cell adhesion to endothelial monolayers: ~60–70% of total Annexin A1 content in human neutrophils was lost in post-adherent cells (Perretti *et al.*, 1996b). More recently, Annexin A1 externalization has been observed also in activated neutrophils, without the need of an endothelial monolayer (Vong *et al.*, 2007). Moreover, a new *mode of secretion* of Annexin A1 from this cell type has been identified via microparticle release (Dalli *et al.*, 2008). Microparticles (also called ectosomes) are organelles that spawn from activated cells by a mechanism involving flippase and scramblase activation, so that their lipid bilayer is inside-out, with phosphatidyl serine being exposed on the outside (Distler *et al.*, 2005). We have found that neutrophil-derived microparticles (Gasser *et al.*, 2003) can display Annexin A1 on their surface, and that this is instrumental to bring about their ability to inhibit neutrophil/endothelium interaction under flow *in vitro* (Dalli *et al.*, 2008). Furthermore, Annexin A1-rich microparticles exerted anti-migratory effects, in line with what has been reported for the protein (see below); it is plausible that mobilization and externalization of Annexin A1 via microparticles or other forms of microvesicles could be a common feature for the release of this protein from different cell sources, similarly to the process of IL-1 secretion (MacKenzie *et al.*, 2001).

Annexin A1 and inflammation

The generation of human recombinant (hr)-Annexin A1, at the time called lipocortin (Wallner *et al.*, 1986), provided great input for the definition of its biological activities, allowing the availability of sufficient amounts for *in vivo* investigations. Annexin A1 was initially characterized for its ability to inhibit prostanoid release (Cirino *et al.*, 1987), an effect that underlined its efficacy in the rat paw oedema model (Cirino *et al.*, 1989). However, hr-Annexin A1 was unable to affect oedema responses elicited by stimuli provoking vasodilatation (e.g. histamine or serotonin), leading to the hypothesis that its mechanism required inhibition of phospholipase A_2 and ensuing reduction in prostaglandin generation. Subsequently it became evident that the anti-inflammatory

properties of hr-Annexin A1 were also relying on important inhibitory effects on the process of leucocyte migration. Movement of blood-borne cells to the site of inflammation, and therefore extravasation into the injured tissue, is a hallmark of the inflammatory response (Ley *et al.*, 2007). Analysis of the effects of Annexin A1 in models of leukocyte migration indicated that this property was not reliant upon prostaglandin generation (Perretti and Flower, 1993), that is Annexin A1 inhibited neutrophil recruitment even when inhibitors of prostaglandin synthesis were without effect. These studies indicated that more than one mechanism of action could be advocated for the pharmacology of Annexin A1 in experimental inflammation.

It therefore soon appeared that the protein was able to produce macroscopic effects due to multiple molecular and cellular actions, in as much as hr-Annexin A1 was able to elicit an anti-pyretic response that was clearly associated with an inhibition of prostaglandin E_2 production in the third ventricle (Carey *et al.*, 1990). These initial observations were then followed up, over the years, by analysis of the tissue protective and anti-inflammatory actions of Annexin A1 using different models in rodents (see Table 1 for a non-exhaustive list of these studies). Efficacy in a given model of pathology could, clearly, guide the potential disease application(s) for Annexin A1 mimetics.

Parallel efforts were devoted to the characterization of the Annexin A1 pharmacophore, noting that the N-terminal region of the protein, which is unique to this protein among the Annexin super family, contains sequences that could reproduce most if not all of the effects of the full protein. Therefore, a peptide spanning the first part of the Annexin A1 N-terminus was synthesized, termed peptide acetyl-2-26 and tested for its ability to inhibit neutrophil recruitment into sites of inflammation (Perretti *et al.*, 1993). This peptide was active in suppressing several aspects of the inflammatory response, including plasma protein extravasation (Perretti *et al.*, 1993; Gavins *et al.*, 2003), nociception (Ferreira *et al.*, 1997) and eliciting, overall, inhibitory effects on neutrohil and monocyte trafficking (Miotla *et al.*, 1995; Getting *et al.*, 1997). It subsequently emerged, perhaps not surprisingly, that in affected tissues the overall tissue-protective effects of peptide acetyl-2-26 might derive from more than one single mechanism; for instance protective actions in myocardial infarct were likely due to a combination of local anti-inflammatory effect (La *et al.*, 2001a,b) as well as of a direct protective action on the cardiomyocyte (Ritchie *et al.*, 2003).

Table 1 lists also the series of *in vivo* experimentations where the effects of peptide acetyl-2-26 have been studied.

Pharmacological properties aside, an important question is the relevance of the pathway centred around endogenous Annexin A1 on the outcome of the host response. Initial experiments addressing this aspect were conducted with neutralizing antisera raised in rabbits or sheep; these antibodies revealed a role for Annexin A1 on nociception (paw pressure test) and inflammatory (leukocyte trafficking, tumour necrosis factor and IL-1 secretion) responses. Moreover, these studies also determined the potential role that Annexin A1 might play in the anti-inflammatory, anti-nociceptive and anti-arthritic effects produced by animal treatment with glucocorticoids (Perretti *et al.*, 1996a; Ferreira *et al.*, 1997; Yang *et al.*, 1999). However, it is evident that a major impetus to this complex aspect – the pathophysiology of Annexin A1 – could be obtained with the generation of Annexin A1 null mice.

Generated by a classical homologues recombination approach by Rod Flower, Bob Hannon *et al.*, these animals bear a transgenic gene that disrupted the endogenous Annexin A1 gene, meanwhile having a LacZ gene under the control of the Annexin A1 promoter (Hannon *et al.*, 2003). Therefore, this important tool could address on the one hand the function of Annexin A1 in a given biological process, and on the other hand determine the potential spatial and temporal regulation of the Annexin A1 gene promoter activity. Indeed, Annexin A1 null mice display an augmented inflammatory reaction and tissue damage when subjected to a given experimental protocol (see Table 2 for a list of experimental settings tested and their major outcome). Moreover, time-dependent induction of the Annexin A1 gene promoter could be monitored in the context of an ongoing inflammatory reaction (Damazo *et al.*, 2005; 2006). In particular, it was noted that extravasated neutrophils bear an activated Annexin A1 gene, possibly affecting the fate of the extravasated leukocytes by promoting apoptosis and phagocytosis of apoptotic cells (Maderna *et al.*, 2005; Scannell *et al.*, 2007). Time-dependent activation of the Annexin A1 gene promoters was observed in cell types other than the neutrophil, including the macrophage, the mast cell and the endothelial cell (Damazo *et al.*, 2006), indicating that this mediator may indeed sustain multiple homeostatic functions. Moreover, systemic inflammation elicited by lipolysaccharide led to marked activation of this gene in lung epithelial cells too (Damazo *et al.*, 2005). The flexibility of this system afforded the possibility to observe a remarkable phenotype in parallel to monitoring gene promoter activity. Absence of Annexin A1 led to animal mortality even when an otherwise non-lethal dose of lipolysaccharide was administered (Damazo *et al.*, 2005), higher degree of cell migration and extravasation into the site of inflammation (Chatterjee *et al.*, 2005; Damazo *et al.*, 2006), higher levels of inflammatory markers in a model of localized joint inflammation (Yang *et al.*, 2004), a faster degree of neurological damage in a model of stroke (Gavins *et al.*, 2007) and a delayed repair in a model of colitis (Babbin *et al.*, 2008) (Table 2).

Annexin A1 target(s)

An important aspect in the biology of Annexin A1, at least in the context of inflammation, has been its mechanism of action, as its solution would have clear pharmacological benefit for drug discovery. Originally thought to act as an inhibitor of phospholipase A_2 (Flower and Blackwell, 1979) with consequent inhibitory effects on the generation of prostaglandin and leukotrienes (Cirino *et al.*, 1987; Flower, 1988; Goulding *et al.*, 1990; 1992; 1996b), the identification of binding sites for Annexin A1 on both peripheral blood neutrophils and monocytes indicated the possible existence of an Annexin A1 receptor(s) (Goulding *et al.*, 1990; 1992; 1996b). Binding sites for the protein have also been reported in both endothelial cells (Srikrishna *et al.*, 2001) and U937 cell line (Solito *et al.*, 2000).

Table 1 Non-exhaustive list of experimental systems where the anti-inflammatory actions of Annexin A1 and its fragments have been analysed

Agent	*Experimental model*	*Observed function*	*References*
Annexin A1	Poly I : C-induced pyrogenesis (rabbit)	↓ Febrile response	Davidson *et al.* (1991)
	Carrageenin paw oedema (rat)	Dose response inhibition	Cirino *et al.* (1989)
	Carrageenin oedema (adrenalectomized rat)	↓ Oedema	Cirino *et al.* (1989)
	Bradykinin-, serotonin-, dextran- or PAF-induced oedema (rat)	Not effective at any dose tested	Cirino *et al.* (1989)
	Compound 48/80 oedema (rat)	↓ Oedema	Cirino *et al.* (1989)
	PLA_2 oedema (rat)	↓ Oedema (~85% at the top dose)	Cirino *et al.* (1989)
	Mesenteric microcirculation activated by zymosan (mouse)	↓ Leukocyte adhesion and emigration (s.c.) ↑ Detachment of adherent neutrophils (i.v.)	Lim *et al.* (1998)
	Heart ischaemia–reperfusion (rat)	↓ Infarct size (≤50%)	D'Amico *et al.* (2000)
	Dorsal injection of polyacrylimide gel (mouse)	↓ PMN migration ↓ PGE_2 and LTB_4 levels ↓ PLA_2 activity	Errasfa and Russo-Marie (1989)
	Cerebral ischaemia–reperfusion (mouse)	↓ Infarct volume, numbers of adherent and rolling leukocytes Improvement of neurological score	Gavins *et al.* (2007)
	IL-1β inflamed air-pouch (mouse)	↓ PMN migration	Perretti and Flower (1993)
	Neutrophil/endothelial interaction under flow (human cells)	↓ PMN adhesion	Hayhoe *et al.* (2006)
	Neutrophil/endothelial interaction (human cells)	↓ PMN transmigration	Walther *et al.* (2000); Zouki *et al.* (2000)
Annexin A1 1-188	Cerebral ischaemia (rat)	↓ Infarct	Relton *et al.* (1991)
	Pyrogenesis caused by central injection of interferon or IL-1β (rat)	↓ Colonic temperature and oxygen consumption	Carey *et al.* (1990)
	Pyrogenesis caused by central injection of PGE_2 (rat)	No change in oxygen consumption or colonic temperature.	Carey *et al.* (1990)
	Lung activation (guinea pig)	↓ TXA_2 induced by bolus injection of LTC_4 or FMLP	Cirino *et al.* (1987)
	IL-1β inflamed air-pouch (mouse)	↓ Leukocyte migration	Perretti *et al.* (1993)
Peptide Ac2-26	IL-1β inflamed air-pouch (mouse)	↓ Leukocyte migration	Perretti *et al.* (1993)
	IL-8 inflamed air-pouch (mouse)	↓ Leukocyte migration	Perretti *et al.* (1993)
	FMLP-induced neutropenia (mouse)	↓ Neutropenia	Perretti *et al.* (1993)
	Albumin extravasation in the skin (mouse)	↓ Skin oedema	Perretti *et al.* (1993)
	Heart ischaemia–reperfusion (rat)	↓ Infarct size by up to 50% ↓ IL-1β and MPO levels in infarcted hearts	La *et al.* (2001a)
	Mesenteric microcirculation activated by ischaemia–reperfusion (mouse)	↓ Leukocyte adhesion and emigration but not rolling ↓ Plasma protein extravasation	Gavins *et al.* (2003)
	Carrageenan paw oedema (rat)	↓ Oedema	Cirino *et al.* (1993)
	Carrageenan-induced arthritis (rat)	↓ The disease severity (intra-articular injection)	Yang *et al.* (1997)
	Glacial acetic acid-induced gastric ulcers (mouse)	↑ Ulcer healing upon a 4 day treatment	Martin *et al.* (2008)
	Contusive spinal cord injury (rat)	↓ PLA_2 and MPO activities ↓ Glial fibrillary acidic protein (4 weeks post injury) ↑ White matter sparing *in vivo*	Liu *et al.* (2007)
	Metabolic inhibition of cardiac myocytes (rat cells)	↓ Cellular injury	Ritchie *et al.* (2005)
	Ovalbumin-induced pleurisy (rat)	↓ Mast cell degranulation and plasma protein leakage ↓ PMN and eosinophil accumulation ↓ Eotaxin release in exudates	Bandeira-Melo *et al.* (2005)
	Splanchnic artery ischaemia–reperfusion (rat)	↓ The progressive fall in blood pressure ↓ PMN accumulation ↓ Bowel injury	Cuzzocrea *et al.* (1997)
	Glycogen-induced peritonitis (mouse)	↓ PMN accumulation	Teixeira *et al.* (1998)
	Ovalbumin-induced sensitization (mouse)	No effect on skin eosinophil recruitment	Teixeira *et al.* (1998)
	Zymosan-induced peritonitis (mouse)	↓ PMN migration (4 h) ↓ Monocyte migration (24 h)	Getting *et al.* (1997)
	In vitro model of septic shock (rat heart)	Abrogation of the fall in the inotropic response to isoprenaline ↓ COX-2 mRNA No effect NOS-2 mRNA	Ritchie *et al.* (2003)
	Mesenteric microcirculation activated by zymosan (mouse)	↓ Leukocyte adhesion and emigration (s.c.) ↑ Detachment of adherent leukocytes (i.v.)	Lim *et al.* (1998)
	Intestinal ischaemia–reperfusion (mouse)	↓ Tissue injury ↓ TNF-α levels ↓ Lethality	Souza *et al.* (2007)
	Neutrophil/endothelial interaction under flow (human cells)	↓ PMN adhesion	Hayhoe *et al.* (2006)
	Phagocytosis of apoptotic neutrophils (human cells)	↑ Clearing by macrophages	Maderna *et al.* (2005)
Peptide Ac2-12	Zymosan inflamed air-pouch (mouse)	↓ PMN recruitment	Perretti *et al.* (2002)
	Heart ischaemia–reperfusion (rat)	↓ Infarct size (≤35%)	La *et al.* (2001a)

COX, cyclooxygenase; FMLP, formyl-Met-Leu-Phe; IL, interleukin; LPS, lipopolysaccharide; LT, leukotriene; MPO, myeloperoxydase; NOS, nitric oxide synthase; PAF, platelet-activating factor; PL, phospholipase; PMN, polymorphonuclear leukocyte; PG, prostaglandin; TNF, tumour necrosis factor; TX, thromboxane.

Table 2 Lessons from the Annexin A1 null mouse

Experimental model	*Outcome*	*References*
Antigen-induced arthritis	↑ Pannus formation (day 7) ↑ Synovial cytokine mRNAs	Yang *et al.* (2004)
Stroke	↑ Neurological score ↑ Cell adhesion (pia mater vessels) ↑ Brain cytokine mRNAs	Gavins *et al.* (2007)
Peritonitis	↑ Neutrophil recruitment (4 and 24 h)	Damazo *et al.* (2006)
Lipolysaccharide Endotoxaemia	Mortality ↑ TNF/IL-6 dysfunctional production from macrophages ↑ Markers of organ injury ↑ Organ infiltration with PMN ↑ Peritoneal trafficking of leukocytes	Damazo *et al.*, (2005)
Paw oedema	↑ Paw volume (selected time points)	Hannon *et al.* (2003)
Cremaster microcirculation	↑ Cell emigration (PAF or zymosan-induced)	Chatterjee *et al.* (2005)
DSS Colitis	↑ Susceptibility to DSS Delayed resolution of the colitis ↓ Fpr2 induction	Babbin *et al.* (2008)

Reported are some of the most informative studies conducted with the Annexin A1 null mouse, showing the major outcome with respect to a selection of the markers under observation, and the respective bibliographic reference.
DSS, dodecylsulphate sodium; IL, interleukin; PAF, platelet-activating factor; PMN, polymorphonuclear leukocyte; TNF, tumour necrosis factor.

The breakthrough came through the work of Volker Gerke and his lab, demonstrating that Annexin A1 and Annexin A1-derived peptides could produce responses in human neutrophils that were blocked by antagonists to the formyl peptide receptor (FPR) (Walther *et al.*, 2000), the so-called Boc derivative (where Boc stands for buthyloxycarbonyl, a bulky group used to protect the N-terminal end of an amino acid sequence during the synthesis of peptides) (Dalpiaz *et al.*, 1999; Paclet *et al.*, 2004). FPR is the receptor for formylated peptides, a G protein-coupled receptor (GPCR) that was cloned in the mid 1980s (Boulay *et al.*, 1990a,b; Becker *et al.*, 1998). The study by Walther *et al.* (2000) demonstrated that addition of Annexin A1 or its peptides to neutrophils reduced the extent of cells transmigration across monolayers of endothelial cells. This effect was paired by a direct activation of the human neutrophils upon application of these peptides in single cell systems, characterized by a transient calcium flux and by shedding of L selectin (Walther *et al.*, 2000).

This fundamental study therefore opened a new avenue of research in the field of annexin A1, allowing further investigations to characterize the functional and molecular links between Annexin A1 and this family of receptors. FPR is the prototype of a family of receptors of which three members have been described in the human system. FPR-like 1 (FPRL-1) and FPR-like 2 (FPRL-2) are structurally related to human FPR although display distinctions with respect to ligand binding: for instance, the original formylated peptide used to clone human FPR, which activates this receptor at low nanomolar concentrations, would activate FPRL-1 at concentrations that are 1000-fold higher, and does not activate FPRL-2 at all (Le *et al.*, 2002; Fu *et al.*, 2006).

Our own studies demonstrated, subsequently, that following adhesion of human neutrophils to endothelial monolayers, endogenous Annexin A1 could be immunoprecipitated together with FPRL-1 (Perretti *et al.*, 2002). Moreover, this receptor was also activated by another endogenous anti-inflammatory mediator, the lipid termed lipoxin A_4; Annexin A1 and lipoxin A_4 compete for binding to this receptor. These data supported the interesting hypothesis that at least two endogenous effectors of anti-inflammation could share a specific GPCR. Because Annexin A1 and lipoxin A_4 have historically been associated to, at least some of, the pharmacological effects produced by glucocorticoids and aspirin, the intriguing idea of this convergence between the most used classes of anti-inflammatory drugs on to this specific target was put forward (Perretti *et al.*, 2002; Gilroy and Perretti, 2005).

The situation is likely more complex as subsequent studies have demonstrated that Annexin A1-derived peptides would activate all three members of the human FPR receptor family (Rescher *et al.*, 2002; Ernst *et al.*, 2004; Karlsson *et al.*, 2005), with not much difference in terms of active concentrations, whereas the full-length protein was shown to bind to FPRL-1 but not to FPR (Hayhoe *et al.*, 2006) (no data are currently available regarding the binding of Annexin A1 to FPRL-2). Altogether, we believe it is plausible that FPRL-1 would be the receptor responsible for transducing the inhibitory signals of Annexin A1 in the pathophysiology of inflammation; hence it could be targeted for novel anti-inflammatory drug discovery programmes (see below).

FPRL-1 is activated by Lipoxin A_4, as stated above, and is characterized by a large degree of promiscuity as it binds to several other peptides, proteins and molecules that are apparently unrelated from a structural point of view (Su *et al.*, 1999; Le *et al.*, 2001; Le *et al.*, 2002; Resnati *et al.*, 2002). This observation that the active site accommodates a series of chemically unrelated agonists is difficult to explain in terms of receptor topology. It remains to be seen which of the agonist/receptor interactions reported in the literature is relevant in the context of an inflammatory reaction. In many studies, for instance, the indication that a given ligand would activate FPRL-1 emerged from individual experiments performed with cells overexpressing this GPCR, with little physiological support. In contrast, the interaction between endogenous Annexin A1 and this receptor, initially demonstrated with human neutrophils, has been confirmed also *in vivo*, at least in the mouse mesenteric tissue (Gastardelo *et al.*, 2009).

This fact brings us to discuss the efforts made to identify the murine receptor(s) responsible for the anti-inflammatory effects of Annexin A1 and its peptides. The mouse FPR receptor family is more complex and difficult to describe, as several genes have been reported for this family at variance from three discovered in the human genome. According to the latest annotation of the mouse genome project data (see UCSC genome browser; mouse chromosome 17 section chr17:18,000,000-18,150,000), FPRL-1 corresponds to the mouse gene previously termed *fpr-rs2* (and now referred to as *fpr2*); therefore the *fpr1* and *fpr2* genes are regarded as unequivocal. The terminology of gene *fpr-rs1* (previously indicated as the orthologue of human FPRL-1) has been changed: it exists in two isoforms, one officially designated *fpr3 (old fpr-rs3)* and the other comprising an exon from *fpr3* and one from the *fpr-rs2*. To add further confusion, genes spanning from fpr-rs3 to fpr-rs7 have been reported in the mouse receptor family. For the purposes of this review, we will restrict our analyses to mouse Fpr1 as the orthologue of the human FPR and to mouse Fpr2 as the orthologue of human FPRL-1.

Following the initial study of Volker Gerke proposing human FPR as the receptor activated by Annexin A1-derived peptides (Walther *et al.*, 2000), a subsequent study was conducted in the mouse taking advantage of mouse Fpr1 knock out animals (Perretti *et al.*, 2001). Absence of the murine orthologue of human FPR indicated that the large majority of the anti-migratory property of peptide acetyl-2-26 was lost in the absence of this receptor when tested using a mouse model of peritonitis. In contrast a good proportion of the inhibitory effect of hr-Annexin A1 was maintained. This indicates that the subsequent observations made for human FPR and human FPRL-1 in terms of their dichotomy in binding abilities to Annexin A1 and its peptides (Hayhoe *et al.*, 2006) was somehow already hinted in the murine system.

The scenario that is currently emerging is that the large majority of properties displayed by hr-Annexin A1 and/or its bioactive peptides are somehow mediated by activation of members of the FPR family. This conclusion is mostly reached through the observation that Boc derivatives, known to act as non-selective antagonists to all members of this family when used at the appropriate concentrations and/or doses (Gavins *et al.*, 2003; Paclet *et al.*, 2004), prevent the expected biological outcomes. This is true for instance in models of myocardial infarct and/or stroke, where the protective effect of the Annexin A1 biologicals was abrogated by co-administration of a Boc derivative (10–50 μg per animal) whereas it was intact in mice nullified for Fpr1 (Gavins *et al.*, 2005; 2007); this would indicate the involvement of a receptor of this family, but not the prototype Fpr1, in bringing about the tissue protective actions of Annexin A1 and its peptides. The same holds true when the effects evoked by Annexin A1 and its peptides on the pituitary (adrenocorticotrophin release) are investigated: Fpr1 deletion did not modify the inhibitory effects produced by the protein (John *et al.*, 2007).

In virtually all systems (with one exception; see below) antagonism of formyl peptide receptors was effective to inhibit a specific action of Annexin A1 and/or its peptides, in the whole animal as well as at the cellular level, in human and murine cells (Walther *et al.*, 2000; Rescher *et al.*, 2002; Ernst *et al.*, 2004). We acknowledge that there is one exception, where the inhibitory signal produced by peptide acetyl-2-26 on human neutrophils was described to arise independently from an interaction with members of the FPR receptor family (only FPR and FPRL-1 are expressed on human granulocytes) (Karlsson *et al.*, 2005). Collectively these studies indicate that receptors of the FPR family are responsible for the majority, if not all, of the effects produced by Annexin A1 in several human and murine biological systems related to inflammatory conditions. The next question would then be whether this information could be of use for the development of novel anti-inflammatory drug discovery programmes.

Annexin A1 receptor for novel drug discovery

Our current view, supported by a series of data produced by our as well as other laboratories, is that one specific receptor of the FPR family conveys the anti-inflammatory signals promoted by Annexin A1 to exert a tonic inhibitory function on the inflammatory reaction. The human system termed FPRL-1, is a GPCR that conveys anti-inflammatory signals promoted also by the Lipoxin A_4 mediator (Chiang *et al.*, 2006), as mentioned above. Nonetheless, other ligands for this receptor including serum amyloid protein A (Su *et al.*, 1999) or fragments of the Beta-amyloid protein (Le *et al.*, 2001), the deposition of which is the cause of Alzheimer's disease, appear to use the same receptor to initiate pro-inflammatory signal. Generations of mice nullified for the orthologue of human FPRL-1 would be one way to assess what function this receptor might have in a defined inflammatory condition. Another way to address the question of whether the *real nature* of FPRL-1, is it pro-inflammatory or anti-inflammatory in nature, is to determine the properties of ligands that are selective for this receptor, without the confounding effect of activating other receptors of this family or other receptors *tout-court*; such ligands have recently been generated.

Amgen have developed a programme to identify small chemical entities that bind and activate selectively FPRL-1 (Burli *et al.*, 2006). At the cellular level, these ligands would promote 'cell activation', that is induce generation of IL-6 from human blood monocytes (Frohn *et al.*, 2007). However, and of great importance, administration of some of these selective FPRL-1 agonists to the experimental animal produced inhibitory effects (ear swelling in response to prostaglandin E_2 and leukotriene B_4 application) (Burli *et al.*, 2006), whereas related molecules able to bind to FPRL-1 without promoting a signalling response were inactive. This prompts us to propose that irrespective of the readouts monitored in 'controlled' cellular systems (often calcium fluxes have been used to monitor FPRL-1 activation upon agonist application), selective ligands to FPRL-1 would produce anti-inflammatory/inhibitory actions in *in* vivo integrated systems.

This conclusion is supported by an even more recent study whereby novel peptides were generated using computer modelling approaches (Shemesh *et al.*, 2008); a peptide that displayed selective binding to FPRL-1, but did not bind to FPR or many other receptors investigated, was identified (Hecht *et al.*, 2009). When given to animals, this peptide was able to inhibit neutrophil trafficking elicited in response to IL-1; of

interest, no *pro-inflammatory* effect was produced by the FPRL-1 peptide ligand once injected into a 6-day-old mouse air-pouch, removing the possibility that – at least in these conditions – Fpr2 activation might convey pro-inflammatory signals and provoke leukocyte trafficking by itself. More importantly, and reminiscent of our own data generated with the Annexin A1 peptide acetyl-2-26 in models of myocardial infarct (La *et al.*, 2001a), this new selective agonist for FPRL-1 was effective in inhibiting heart tissue injury when animals where subjected to an ischaemia–reperfusion procedure (Hecht *et al.*, 2009). Such a protective effect was associated with reduced tissue infiltration by neutrophils, again in agreement with what was reported for hr-Annexin A1 (D'Amico *et al.*, 2000). Therefore, this information is again in line with the notion that the Annexin A1 receptor, FPRL-1 (or ALX if the nomenclature used for lipid receptors – in this case the Lipoxin A_4 receptor – is followed; Chiang *et al.*, 2006), would be a target suitable for identifying selective agonists and develop novel anti-inflammatory therapeutics.

Conclusion

In summary these are exciting times as over 20 years of research on the physiology, pathology and pharmacology of Annexin A1 is now coming together in a coherent and integrated fashion, providing a platform for a promising future whereby this line of research could be effectively exploited for the development of novel anti-inflammatory drugs.

The philosophy that pervades the research approach in our laboratory as well as in many more worldwide suggests that the study of endogenous anti-inflammatory agonists could lead to the identification and development of better anti-inflammatory therapeutics. These drugs should be burdened to a lesser degree by side effects as they will be acting by mimicking the way our body disposes, safely, of the complex inflammatory process (Perretti, 1997; Gilroy *et al.*, 2004; Serhan *et al.*, 2007). A correlated concept we recently put forward in an *announcement article* (which stemmed from a 1 day workshop organized by the British Pharmacological Society in April 2006), and that should be reiterated here, is the need to determine, as part of drug discovery programmes, whether anti-inflammatory therapeutics under development are *resolution-toxic* or resolution-safe. Indeed, virtually 100% of new anti-inflammatory therapeutics are tested for their ability of inhibiting production and/or function of pivotal pro-inflammatory cytokines, whereas their potential modulation of endogenous anti-inflammatory mediators is scarcely, if at all, determined (Serhan *et al.*, 2007). We therefore propose that besides attempting to develop novel therapeutics by mimicking specific effectors of endogenous anti-inflammation, we should also determine if old and new anti-inflammatory drugs are detrimental to the expression and/or function of one or more of these homeostatic effectors [e.g. Annexin A1, Lipoxin A_4, prostaglandin D_2, melanocortins and many more (Perretti, 1997; Gilroy *et al.*, 2004)], hence display some resolution-toxic features that could potentially limit their overall therapeutic efficacy.

Acknowledgements

The work conducted in our laboratory and referred to in this review was funded by the Arthritis Research Campaign UK (senior fellowship 15755), the Wellcome Trust (programmes 069234 and 086867) and the British Heart Foundation (FS/2000076 and FS/03/100/16326). We apologize to the many colleagues whose work could not be quoted here for space limitations.

Conflict of interest

MP declares licensing of a patent on Annexin A1-derived anti-inflammatory peptides to Unigene Corp (Fairfield, NJ, USA). JD, no conflict to declare.

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