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Formyl peptide receptors and the regulation of ACTH secretion: targets for annexin A1, lipoxins, and bacterial peptides

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Abstract

The *N*-formyl peptide receptors (FPRs) are a family of G-protein coupled receptors that respond to proinflammatory *N*-formylated bacterial peptides (e.g., formyl-Met-Leu-Phe, fMLF) and, thus, contribute to the host response to bacterial infection. Paradoxically, a growing body of evidence suggests that some members of this receptor family may also be targets for certain anti-inflammatory molecules, including annexin A1 (ANXA1), which is an important mediator of glucocorticoid (GC) action. To explore further the potential role of FPRs in mediating ANXA1 actions, we have focused on the pituitary gland, where ANXA1 has a well-defined role as a cell-cell mediator of the inhibitory effects of GCs on the secretion of corticotrophin (ACTH), and used molecular, genetic, and pharmacological approaches to address the question in well-established rodent models. Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis identified mRNAs for four FPR family members in the mouse anterior pituitary gland, Fpr-rs1, Fpr-rs2, Fpr-rs6, and Fpr-rs7. Functional studies confirmed that, like dexamethasone, ANXA1 and two ANXA1-derived peptides (ANXA1₁₋₁₈₈ and ANXA1_{Ac2-26}) inhibit the evoked release of ACTH from rodent anterior pituitary tissue *in vitro*. Fpr1 gene deletion failed to modify the pituitary responses to dexamethasone or ANXA1_{Ac2-26}. However, lipoxin A4 (LXA4, 0.02–2 μM, a lipid mediator with high affinity for Fpr-rs1) mimicked the inhibitory effects of ANXA1 on ACTH release as also did fMLF in high (1–100 μM) but not lower (10–100 nM) concentrations. Additionally, a nonselective FPR antagonist (Boc1, 100 μM) overcame the effects of dexamethasone, ANXA1₁₋₁₈₈, ANXA1_{Ac2-26}, fMLF, and LXA4 on ACTH release, although at a lower concentration (50 μM), it was without effect. Together, the results suggest that the actions of ANXA1 in the pituitary gland are independent of Fpr1 but may involve other FPR family members, in particular, Fpr-rs1 or a closely related receptor. They thus provide the first evidence for a role of the FPR family in the regulation of neuroendocrine function. —John, C. D., Sahni, V., Mehet, D., Morris, J. F., Christian, H. C., Perretti, M., Flower, R. J., Solito E., Buckingham J. C. Formyl peptide receptors and the regulation of ACTH secretion: targets for annexin A1, lipoxins, and bacterial peptides.

Keywords

glucocorticoids; annexin I; pituitary; HPA axis; formyl peptides; lipoxins

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THE HYPOTHALAMO-PITUITARY-ADRENOCORTICAL (HPA) axis plays an essential role in the maintenance of homeostasis. In normal circumstances its activity is tightly regulated with obvious changes in glucocorticoid (GC) secretion occurring only in accord with the circadian rhythm and in conditions of stress. However, subtle disturbances in GC secretion and/or activity are not uncommon in clinical medicine and are increasingly linked to the pathogenesis of diseases, which are endemic in the Western world, *e.g.*, obesity, hypertension, and depression. Such disturbances can be brought about by a variety of mechanisms, including impairments of the negative feedback regulation of the axis by glucocorticoids, as occurs in depression and other mental health disorders (1,2).

We have previously demonstrated that the feedback actions of GCs on HPA function are mediated in part by a 37-kDa protein, annexin 1 (ANXA1, also known as lipocortin 1) (3). ANXA1 is a well-characterized member of the annexin family of Ca^{2+} - and phospholipid-binding proteins. It is strongly expressed in the neuroendocrine system, particularly in the anterior pituitary gland and in specific loci in the hypothalamus, notably the median eminence and cells surrounding the third ventricle (4-6). The last decade has seen increased understanding of the molecular basis of ANXA1 action in the pituitary gland where it is now apparent that it acts as a paracrine/juxtacrine modulator of hormone release (7). ANXA1 is expressed mainly in the nonendocrine folliculostellate (FS) cells of the pituitary, particularly at points where the FS cells make contact with the endocrine cells (8,9). GCs augment ANXA1 gene expression in these cells and promote the translocation of preformed ANXA1 from the cytoplasm to the outer surface of the plasma membrane (5,10). The cell surface form of the protein is serine-phosphorylated, a modification that appears essential both for the translocation of the protein across the membrane and for the biological activity of the protein (11,12). Binding and functional studies suggest that the exportation of ANXA1 from FS cells enables the protein to gain access to specific, high-affinity, membrane-bound binding sites (putative receptors) on the surface of the pituitary endocrine cells (13) and thereby suppress peptide hormone release (7). At present, little is known of the nature of these putative "receptors" or of the signaling mechanisms they use to inhibit hormone release. However, recent studies in other systems, and particularly in the host defense system, have indicated that members of the formyl peptide receptor (FPR) family may be involved in mediating at least some of the actions of ANXA1 (14-16).

The FPR family comprises a group of seven transmembrane G-protein coupled receptors. It has a complex evolutionary history and the number of family members varies significantly between species. In the human, three members have been identified, each encoded by a different gene. These were termed FPR, FPR-like1 (FPRL1), and FPRL2 (17). However, a recent review by the International Union of Pharmacology Nomenclature Commission concluded that FPRL1 should be termed ALX to reflect the fact that the endogenous lipid mediators, lipoxin A_4 (LXA_4) and the aspirin-triggered 15-*epi*- LXA_4 (ATL) are the most potent and selective agonists of this receptor reported to date (18). In the mouse, the FPR gene cluster is expanded to encode eight receptors termed Fpr1 and the FPR-related receptors: Fpr-rs1 - Fpr-rs7 (19, 20). The sequences of these receptors are available on GenBank, although it should be noted that some controversy has arisen over the sequence of Fpr-rs1 (19-21), which may be explained by strain-specific polymorphisms (19), and accordingly, the sequence has been temporarily removed from the database for review. The precise relationship of the various murine Fpr receptors to the human FPR family requires further definition, but current evidence suggests that Fpr1 is equivalent to the human FPR (amino acid sequence identity of 76%) and Fpr-rs1 and Fpr-rs2 are broadly comparable to ALX (amino acid sequence identity of 74% and 76%, respectively) (22).

The human FPR and murine Fpr1 show high affinity for N-formyl peptides (*e.g.*, fMLF, (22). Since the principal natural source of such peptides is bacteria and FPRs are expressed in

abundance by cells of the host defense system (*e.g.*, neutrophils), a view emerged that the FPR family had evolved as chemoattractant receptors that assist the organism in countering bacterial infections, in particular, by facilitating the trafficking of phagocytes to the site of bacterial invasion (reviewed in 23,24). However, the discovery that formylated peptides are also produced by mitochondria (25) together with evidence that members of this receptor family are also sensitive to some nonformylated peptides (*e.g.*, HIV peptides, β -amyloid; reviewed in (26)) and are expressed in a variety of cells/tissues has raised the possibility that FPRs have far more diverse and complex roles in biology.

ANXA1 was first identified as a putative endogenous ligand of FPR in granulocytes (16). Subsequent studies revealed that peptides derived from its N-terminal can activate all members of the human FPR family (27) and may also interact with the murine Fpr1 and a further lipoxin-sensitive member of the murine Fpr family (14) but that the full-length protein shows selectivity for ALX and its murine homologs (28). These findings raise the possibility that one or more members of the FPR family may mediate the powerful inhibitory effects of ANXA1 on ACTH release in the anterior pituitary gland and, thereby, play a critical role in effecting the negative feedback actions of glucocorticoids. In addition, by responding to bacterial and viral peptides, the receptors may contribute to the neuroendocrine responses to bacterial endotoxemia and viral infections. In the present study, we have addressed these hypotheses by examining 1) the effects of two selective chemically unrelated agonists (fMLF and lipoxin A4) and two antagonists (Boc1 and Boc2) of these receptors on the release of ACTH from rodent anterior pituitary tissue, 2) the integrity of ANXA1-dependent GC actions in pituitary tissue from Fpr1-null mice and 3) the expression of Fprs in murine pituitary tissue. Our results mitigate against a role for Fpr1 but suggest that other Fpr family members, in particular Fpr-rs1, may play an important role in the regulation of ACTH secretion and, hence, in the maintenance of homeostasis.

MATERIALS AND METHODS

Animals

Adult male rats and mice were housed in rooms with controlled lighting (lights on 0800-2000), in which the temperature was maintained at 21-23°C, and food and water were available *ad libitum* for at least a week before the experiments were performed. The rats (Sprague-Dawley strain), weighing $\sim 200 \pm 20$ g, were purchased from Harlan Olac (Banbury, UK). Mice (25 ± 4 g) in which the Fpr1 gene was deleted (FPR1 KO mice) and backcrossed with C57/BL/6 for 6 generations (29), were bred in-house. Weight-matched male C57BL/6 mice (purchased from Banton and Kingsman, Hull, UK) were used as wild-type (WT) controls. All procedures were carried out under license in accord with the UK Animals (Scientific Procedures) Act, 1986.

Materials

The following were used: dexamethasone sodium phosphate (David Bull Laboratories, Warwick, UK); human recombinant ANXA1₁₋₁₈₈ (prepared by expression in *Escherichia coli* (30) (gift from Dr. F. Carey, Astra-Zeneca, Macclesfield, UK); an N-terminal ANXA1 peptide ANXA1_{Ac2-26} (custom-made in-house by Dr. Ian Moss, Advanced Biotechnology Centre, Imperial College London; purity of the product was verified by mass spectrometry and HPLC), which was first dissolved in small amounts of 1 M NH₄HCO₃ and then diluted in medium (the final concentration of NH₄HCO₃ never exceeded 20 mM, and appropriate controls were included in all experiments); FPR agonists, fMLF (Sigma Chemical, Poole, Dorset, UK); synthetic lipoxin A4 (5S,6R,15S-trihydroxy-7,9,13-trans-11-cis-eicosatetraenoic acid, LXA4, Calbiochem, Nottingham, UK); the FPR antagonists, N-*tert*-butoxy-carbonyl-methionyl-leucyl-phenylalanine (Boc1) and N-*tert*-butoxy-carbonyl-phenylalanine-leucyl-phenylalanine-leucyl-phenylalanine (Boc2), both from ICN Pharmaceuticals (Basingstoke,

UK). Boc compounds were first dissolved in small amounts of DMSO and then diluted in medium (the final concentration of DMSO never exceeded 1%, and appropriate controls were included in all experiments); Forskolin (Sigma, UK) was first dissolved in small amounts of ethanol and then diluted in medium; the final concentration of ethanol never exceeded 0.1%, and appropriate controls were included in all experiments. The concentrations of ANXA1₁₋₁₈₈ (27 pM) ANXA1_{Ac2-26} (6.8 μ M), dexamethasone (100 nM) and forskolin (100 μ M) used were based on our published findings (5,7,11) and took into account the striking differences in potency of the two ANXA1 peptides in our system. Concentrations of Boc1 and Boc2 were selected on the basis of preliminary experiments and information in the literature.

Incubation of anterior pituitary tissue

Anterior pituitary glands, removed from rats or mice immediately after decapitation, were divided into segments of approximately equal size. The segments were distributed randomly (1 segment per cell culture insert) in 12-well tissue culture plates (Costar, Cambridge, MA, USA) containing 2 ml Earle's balanced salt solution (EBSS, Sigma Chemical) enriched with aprotonin (1%, Bayer UK Ltd.), pH 7.4. The plates were incubated for 2 h at 37°C in a humidified atmosphere saturated with 95% O₂/5% CO₂ gas with medium changes at 1 h. The segments were then transferred to fresh medium containing the adenylyl cyclase activator, forskolin (Sigma) or, in the case of controls, an equal volume (2 ml) of medium alone and incubated for a further 1 h. Where appropriate, drugs (dexamethasone, recombinant annexin peptides, FPR agonists/antagonists) were included throughout the preincubation and final incubation periods. The medium from the final incubation was collected and stored in aliquots at -20°C for subsequent immunoassay. The pituitary segments were weighed on a torsion balance and discarded.

RIA of ACTH

ACTH was determined in duplicate using a modification of a double antibody (Ab) method (31) with a well-characterized primary Ab raised in the rabbit against human ACTH₁₋₃₉, (code: AFP6328031 National Hormone and Pituitary Programme (NIDDK), Torrance, CA), human ACTH₁₋₃₉ as a standard (NIDDK) and ¹²⁵I-labeled ACTH₁₋₃₉ as the tracer. Separation of the bound and free peptide was achieved by sheep anti-rabbit IgG-coated beads (Amersham Pharmacia, Little Chalfont, Buckinghamshire, UK). Inter- and intra-assay variations were 11.1% and 10.7%, respectively. Samples from individual experiments were always assayed in single batches.

Reverse transcriptase-polymerase chain reaction

As members of the FPR family show a high degree of sequence homology, we used reverse transcriptase-polymerase chain reaction (RT-PCR) analysis with gene-specific primers to detect mRNAs for these receptors in mouse anterior pituitary and hypothalamic tissue. Briefly, the animals were killed by CO₂ overdose and perfused *via* the left ventricle with 25 ml sterile physiological saline (Phoenix Pharma, Gloucester, UK) for 10 min. Anterior pituitary and hypothalamic tissue was then collected. Total RNA was isolated using a spin column, according to manufacturer's instructions (RNeasy kit, Qiagen, Crawley, UK) and reverse transcribed with 2 μ g oligo(dT) 15 primer (Promega, Southampton, UK), 10 U avian myeloblastosis virus (AMV) reverse transcriptase, 40 U ribonuclease inhibitor (Promega) and 1.25 mM of each deoxyribonucleoside triphosphate (dNTP) for 20 min at 42°C. The resultant cDNA was used for PCR using published primer sequences (19) for murine Fpr1, Fpr-rs1, Fpr-rs2, Fpr-rs6, Fpr-rs-7, and GAPDH, and in separate experiments, primers that covered the open reading frame (Table 1; (19)). PCR analysis was performed on a TouchDown thermal cycler exactly as reported previously (19) (Thermo-Hybaidd, Ashford, UK).

Direct sequencing of the PCR products was performed by the Advanced Biotechnology Centre (Imperial College, London) and subsequently confirmed by standard nucleotide basic local alignment search tool (BLAST) using the sequence data for Fpr1, Fpr-rs1 Fpr-rs2, Fpr-rs6, and Fpr-rs7 listed in GenBank under accession numbers NM_013521, NM_008038, NM_0080039, NM_177316, and NM_1777317, respectively (19).

Data analysis

Preliminary analysis by the Shapiro and Wilks test showed that the data from the *in vitro* studies were normally distributed. Subsequent analysis was done by ANOVA with *post hoc* comparisons by Duncan's multiple range test. Differences were considered to be significant if $P < 0.05$. As the basal rate of anterior pituitary hormone release varied between experiments *in vitro*, statistical comparisons were made only within experiments.

RESULTS

Effects of fMLF and lipoxin A4 on ACTH release *in vitro*

Initial studies examined the effects of the two FPR agonists, the *N*-formyl peptide, formyl-methionineleucyl-phenylalanine (fMLF), and lipoxin A4 (LXA4), on the resting and forskolin-stimulated release of ACTH from rat pituitary tissue *in vitro*. Forskolin, an adenylyl cyclase activator, was selected as a secretagogue because the regulatory actions of ANXA1 on ACTH release occur at a point distal to the generation of cyclic AMP (the principal mediator of CRH-induced ACTH secretion), PKA activation, and Ca^{2+} entry (5,11). Forskolin (100 μM) caused a significant increase in ACTH release ($P < 0.01$ vs. basal), which was reversed by dexamethasone (100 nM, positive control, $P < 0.01$ vs. forskolin, Fig. 1A-C). At low concentrations, fMLF (10 and 100 nM), which shows a relatively high affinity for the murine Fpr1 vs. other FPR family members (22), had no effect on basal ACTH release or on the capacity of forskolin to stimulate ACTH release (Fig. 1A). At higher concentrations (1-100 μM), however, it behaved like dexamethasone and blocked the secretory responses to forskolin ($P < 0.01$) without affecting basal ACTH release (Fig. 1B). LXA4 (20 nM–2 μM), which shows selectivity for human ALX and binds with high affinity ($K_d = 1.5$ nM) to a murine receptor with 97% homology to Fpr-rs1 (19,21), also blocked the secretory responses to forskolin ($P < 0.01$ vs. forskolin) and was considerably more potent than fMLF in this regard. At low concentrations (20 and 200 nM), LXA4 had no effect on basal ACTH release but at the highest concentration tested (2 μM), it increased ACTH release ($P < 0.01$ vs. basal, Fig. 1C).

Effects of FPR blockade on the ability of dexamethasone, ANXA1, fMLF and lipoxin A4 to suppress ACTH release *in vitro*

Previous studies have demonstrated the ability of certain *N*-*tert*-butoxy-carbonyl-peptides (Boc-peptides) to inhibit FPR function, although the specificity of these antagonists for the various receptor subtypes is unclear. Here, we describe the effects of two Boc-peptides, Boc1 (Figs. 2 and 3) and Boc2 (Fig. 4), on the ability of fMLF, lipoxin A4, dexamethasone, and two ANXA1-derived peptides (ANXA1₁₋₁₈₈ and ANXA1_{Ac2-26}) to suppress forskolin-stimulated ACTH release from rat pituitary tissue *in vitro*. At both concentrations tested (50 and 100 μM), Boc1 had no effect on the basal or forskolin-stimulated release of ACTH. At the lower concentration (50 μM), it also failed to modify the significant ($P < 0.01$ vs. forskolin) inhibitory effects of dexamethasone (100 nM, Fig. 2A), ANXA1₁₋₁₈₈ (27 pM, Fig. 2B) or ANXA1_{Ac2-26} (6.8 μM , Fig. 2C). By contrast, at the higher concentration tested (100 μM), Boc1 fully overcame the suppressive effects of dexamethasone (Fig. 2A), both ANXA1-derived peptides (Figs. 2B, C), fMLF (10 μM) and lipoxin A4 (0.2 μM , Fig. 3).

The second Boc-derivative tested, Boc2 (100 μM), produced a very different profile of data (Fig. 4). Alone, this compound caused a small but significant inhibition of basal ACTH release

($P < 0.05$ vs. basal). It also suppressed the secretory response to forskolin ($P < 0.01$ vs. forskolin alone) but did not influence the suppressive effects of dexamethasone on forskolin-stimulated ACTH release ($P > 0.05$ forskolin + dexamethasone vs. forskolin + dexamethasone + Boc2).

Effects of FPR1 gene deletion on the ability of dexamethasone and ANXA1 to suppress ACTH release *in vitro*

Pituitary tissue from WT C57Bl6 mice responded to forskolin (100 μ M) *in vitro* with an increase in ACTH release, which was suppressed by dexamethasone (100 μ M, Fig. 5A) and ANXA1_{Ac2-26} (6.8 μ M, Fig. 5B). Basal ACTH was higher in tissue from Fpr1 KO mice ($P < 0.05$, WT vs. KO), although the incremental responses to forskolin were similar in both mouse strains. The responses of the Fpr1 KO tissue to forskolin were readily blocked by dexamethasone (100 nM) and ANXA1_{Ac2-26} (6.8 μ M), as they were in the WT control ($P < 0.01$).

Analysis of FPR transcripts in mouse anterior pituitary tissue

The functional data presented above exclude a role for Fpr1 in mediating the inhibitory effects of ANXA1 on ACTH secretion but support the premise that other members of the FPR family may play a significant role. We therefore performed RT-PCR on pituitary tissue from WT mice to detect and identify the expression of Fpr transcripts. The data are shown in Fig. 6. In all cases, the sequences of the amplified products corresponded with those reported by Wang and Ye (2002) (19). Fpr1 mRNA was not detectable in the mouse pituitary gland with 30 amplification cycles (Fig. 6A) using either the published primer sequences (19) or sequences that cover the open reading frame (data not shown, see Table 1 for primer details). However, when the amplification was increased to 40 PCR cycles, a weak signal for Fpr1 emerged (Fig. 6B). Two clear bands of approximately equal intensity which corresponded to Fpr-rs1 and Fpr-rs2 were evident at both levels of amplification (30 or 40 PCR cycles, note the DNA sequence for Fpr-rs1 is under review). In addition, both Fpr-rs6 and Fpr-rs7 were strongly expressed in the anterior pituitary gland. All transcripts probed, including Fpr1, were readily detected in mouse hypothalamic tissue, which was used as a control, using 30 or 40 amplification cycles (Fig. 6C, D). The differential expression profile of FPR genes described here in the neuroendocrine system and elsewhere (19) implies that, despite their high sequence homology, the genes are differentially regulated.

DISCUSSION

The present study used genetic, pharmacological, and molecular approaches to examine the role of the G-protein-coupled FPR family in mediating the inhibitory actions of ANXA1 on anterior pituitary hormone release. Our results exclude a role for Fpr1 but provide important novel evidence to suggest that other FPR family members, in particular, Fpr-rs1, are functionally important in this regard. They thus provide the first evidence for a role of the FPR family in the regulation of neuroendocrine function and peptide release.

Our previous studies demonstrated that ANXA1 plays a pivotal role in the neuroendocrine system as a mediator of the acute inhibitory effects of glucocorticoids on hormone secretion (5,11). They also provided evidence that within the pituitary gland ANXA1 acts *via* a paracrine/juxtacrine mechanism and that its suppressive effects on the release of ACTH and other pituitary hormones are effected *via* receptors located on the surface of the endocrine cells (13). Walther and colleagues were the first to suggest that the formyl peptide receptor (FPR) may be an important target for ANXA1 in the human host defense system (16). However, subsequent findings on human tissue indicate that FPR is not the sole, or even the major, ANXA1 receptor and that other FPR family members may play a significant role (14,27,28,

32). Similar conclusions emerged from a recent study on mice that explored the cardioprotective actions of ANXA1 (33).

The present data mitigate strongly against a role for Fpr1, the murine equivalent of the human FPR, as a target for ANXA1 action in the rodent anterior pituitary gland. First, fMLF, which shows some selectivity for the human FPR ($K_d \sim 3$ nM) and murine Fpr1 ($EC_{50} = 5$ nM) (34, 35), had no effect on basal or forskolin-stimulated ACTH when applied to rat tissue in low concentrations that would be expected to activate these receptors and, indeed, are known to elicit chemotactic and antimicrobial responses in human neutrophils (36,37). Secondly, the inhibitory effects of dexamethasone and the ANXA1-derived peptides, ANXA1₁₋₁₈₈ and ANXA1_{Ac2-26}, on forskolin-evoked ACTH release were unaffected by the FPR antagonist Boc1 at a concentration (50 μ M) above that necessary to block human FPR and murine Fpr1 in other systems [20 nM–20 μ M (16,38,39)]. Third, pituitary tissue from Fpr1-null mice responded normally to both dexamethasone and ANXA1_{Ac2-26}. Fourth, the full-length ANXA1 molecule, which readily suppresses ACTH release in our preparation (5) does not bind to FPR in human cells/tissues (32). Finally, and in accordance with expression data from normal human anterior pituitary tissue (40), our RT-PCR results do not support a role for Fpr1. Thus, we were unable to detect Fpr1 mRNA in murine pituitary tissue using 30 PCR cycles, although a weak band was apparent with 40 amplification cycles, while in the rat, no activity was detected at either level of amplification (data not shown). This contrasts with the murine hypothalamus, where Fpr1 mRNA was readily detected. The functional significance of the weak band detected in the mouse pituitary at such a high level of amplification (40 PCR cycles) is questionable and, indeed, it is conceivable that the signal originated from a residual blood leukocyte not flushed out by the perfusion process. Nonetheless, it should be noted that pituitary tissue from Fpr1-null mice showed an increase in basal ACTH release *vs.* the WT counterpart. The reason for this is unclear, but because in acute experiments the basal output of ACTH from pituitary tissue *in vitro* reflects the degree of hypothalamic drive to the tissue prior to autopsy (41), it is possible that Fpr1 contributes directly or indirectly to the regulation of CRH/AVP secretion. This possibility warrants further exploration.

Determination of the potential role of other FPR family members in effecting the responses of ANXA1 is hindered by the lack of specific antibodies and ligands for these receptors. Nonetheless, several lines of evidence are consistent with a potential role for Fpr-rs1 or a closely related receptor. Fpr-rs1 and Fpr-rs2 have been described as the murine orthologs of the human ALX, which binds and responds to lipoxins, ANXA1, and various ANXA1 peptides (19,21,22). While there is general agreement that Fpr-rs2 functions as a low-affinity proinflammatory receptor (EC_{50} for fMLF = 5.3 μ M), (22,42), Hartt *et al.* (22) argued that Fpr-rs1 may fulfill an anti-inflammatory role. Such a view is supported by evidence that the anti-inflammatory eicosanoid, lipoxin A4, binds with high affinity ($K_d = 1.5 \pm 0.6$ nM) to a receptor (LXA₄R) with very high sequence homology (97%) to Fpr-rs1 (19,21). As demonstrated by RT-PCR analysis, Fpr-rs1 and Fpr-rs2 are both expressed in the mouse pituitary gland. In addition, while the expression profile in the rat is more difficult to define at this stage as the sequences of rat Fpr-rs1/Fpr-rs2 await confirmation, using murine primers directed against well-conserved sequences, we detected clear bands corresponding to Fpr-rs1 and Fpr-rs2 mRNAs by RT-PCR in the rat pituitary gland (data not shown). Our functional data revealed that the inhibitory effects of dexamethasone, ANXA1, and ANXA1 peptides on forskolin-stimulated ACTH release are mimicked by lipoxin A4 and that lipoxin A4 is effective at concentrations in the nanomolar range, which would readily activate the Fpr-rs1-like receptor cloned by Takano and colleagues (21). The inhibitory responses observed with high concentrations of fMLF may also be effected *via* a similar mechanism although, while fMLF is a low potency agonist of Fpr-rs2 (22), its actions at Fpr-rs1 have yet to be characterized. Our finding that the nonselective FPR antagonist Boc1 reversed the inhibitory effects of fMLF, lipoxin A4, dexamethasone, and ANXA1_{Ac2-26} on the evoked release of ACTH, albeit only in

a high concentration, is also compatible with our proposal that ANXA1 may act *via* Fpr-rs1 or a closely related receptor, although it should be noted that the bulky *N-tert*-butyloxycarbonyl group contained within this molecule is known to interact with several members of the FPR family (14,32). Interestingly, previous studies have demonstrated additive effects of dexamethasone and aspirin in regulating acute inflammation, which have been ascribed to the aspirin-induced formation of 15-epi-lipoxin A4 (ATL). Similarly, ATL and ANXA1 (which is induced by dexamethasone) have been shown to act in concert at the ALX to inhibit polymorphonuclear cell recruitment to sites of inflammation (32). Several studies have shown that lipoxin A4 and 15-epi-lipoxin A4 are generated in the rodent brain and have neuroprotective properties (43,44). If ATL synthesis is similarly induced by aspirin in neuroendocrine tissues, then it is possible that glucocorticoids and aspirin will also have additive effects on ACTH release, although it must be noted that the data from such studies will be complicated by the concurrent aspirin-induced blockade of the synthesis of prostaglandins and thromboxane, both of which are inhibitory to ACTH release (45).

If, as the present data indicate, the actions of ANXA1 in the pituitary gland are effected *via* an FPR member, possibly Fpr-rs1, it is pertinent to give some consideration to the signaling system that mediates this effect (Fig. 7). CRH and forskolin-evoked ACTH release is dependent on the generation of cyclic adenosine monophosphate (cyclic AMP), activation of cyclic AMP-dependent kinase (PKA), and subsequent influx of Ca^{2+} via L- Ca^{2+} channels which, in turn, triggers vesicle fusion and exocytosis (5,46). Our previous data have shown that the signaling sequence evoked by ANXA1 prevents exocytosis by disrupting signaling at a point distal to Ca^{2+} entry (5,11). Indeed, the inhibitory effects of ANXA1 in the pituitary are most pronounced when Ca^{2+} ionophores are used to elicit ACTH release (5). Consistent with these data, FPR family members signal *via* heterotrimeric pertussis toxin-sensitive Gi proteins (17) and Gi activation has been shown to exert an inhibitory influence on cyclic AMP-driven ACTH release in the AtT20 cell line (46,47). It is thus conceivable that FPR-dependent Gi activation is the first step in the signaling cascade used by ANXA1. How Gi activation depresses the ACTH release is unclear, but it is worth noting that FPRL1 activation has been shown to induce actin polymerization in leukocytes (48), a process known to inhibit vesicle fusion in endocrine cells via mechanisms involving activation of small G-proteins (rho/rac), as also have glucocorticoids in pituitary tissue (49).

Three further observations reported here also merit discussion, namely, the apparent stimulatory effects of lipoxin A4 in micromolar concentrations on basal ACTH release, the inhibitory effect of Boc2 on the resting and evoked release of ACTH and the expression of further members of the FPR family in the pituitary gland, in particular, Fpr-rs6 and Fpr-rs7. With regard to the positive action of lipoxin A4, note that in high concentrations, ANXA1 itself (like lipoxin A4) has been shown to initiate basal ACTH release but to prevent the further release of the hormone in response to secretagogues such as CRH and forskolin (5). Interestingly, a similar paradox has been recognized in the host defense system in which ANXA1 is capable of both inducing and suppressing neutrophil chemotactic activity, apparently *via* members of the FPR family (14,21). The mechanism by which ANXA1 elicits ACTH secretion is not understood, but a positive role for ANXA1 in the processes regulating exocytosis has also been described in the pancreas (50,51). Our further observation that Boc2 depresses both basal and forskolin-stimulated ACTH release raises the possibility that a member of the FPR family contributes to the processes facilitating ACTH secretion. Although Boc2 is reputed to show some selectivity for Fpr1, our RT-PCR and functional data mitigate against a role for this family member. Potential candidates are Fpr-rs6 or Fpr-rs7, which are not activated by classical FPR ligands (*e.g.*, fMLF, WKYMVm, (19) but are prevalent in the pituitary gland.

In conclusion, disturbances in HPA function, and hence cortisol secretion, are strongly implicated in the pathogenesis of a number of diseases that are endemic in the Western world (*e.g.*, depression, type 2 diabetes, and hypertension, reviewed in 2). Because a growing body of evidence that suggests that disruption of glucocorticoid feedback is a major cause of hypercortisolaemia, our investigation of the potential role of the FPR family in mediating the ANXA1-dependent acute inhibitory actions of GCs on HPA function is particularly timely and important. Our data show for the first time that several members of the FPR receptor family are expressed in the rodent hypothalamus and pituitary gland. In addition, they provide novel evidence to support the premise that Fpr-rs1, or a closely related receptor, is an important target for ANXA1 in the neuroendocrine system. They thus represent a significant advance in our understanding of the complex mechanisms by which GCs exert their negative feedback actions in the neuroendocrine system. The data also raise the important questions as to the role of the FPR family in mediating neuroendocrine responses to bacterial/viral infections and to locally generated antiinflammatory eicosanoids (*e.g.*, lipoxins) and, hence in maintaining homeostasis. We, therefore, consider that our study has not only generated novel and important information about the mechanisms of glucocorticoid feedback but also paved the way for further exploration of the roles of this complex receptor family and its various endogenous and exogenous ligands in effecting the neuroendocrine responses that are critical to homeostasis in health and disease.

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Figure 1.

Comparison of the effects of graded concentrations of the formyl peptide (A), fMLF (B), and lipoxin A4 (C) on the resting and forskolin-stimulated release of ACTH from rat pituitary tissue *in vitro*. Dexamethasone (100 nM) was included as a positive control in each experiment. Open columns, basal; solid columns, forskolin (100 μM). The data are the mean ± SEM ($n=8$). ** $P < 0.001$ vs. basal; † $P < 0.05$; †† $P < 0.01$ vs. forskolin alone (ANOVA plus Duncan's multiple-range test).

Figure 2.

Effects of an FPR antagonist, Boc1, on the ability of dexamethasone (A; 100 nM), ANXA1₁₋₁₈₈ (B; 27 pM), and ANXA1_{Ac2-26} (C) (6.8 μM) to suppress the release of ACTH from rat pituitary tissue *in vitro* evoked by forskolin (100 μM). Open columns, Boc1-free; closed columns, Boc1 (50 μM); hatched columns, Boc1 (100 μM). ** $P < 0.01$ vs. basal; †† $P < 0.001$ vs. forskolin alone (ANOVA plus Duncan's multiple range test, $n=8$).

Figure 3.

Effects of the FPR antagonist, Boc1 (100 µM), in the presence and absence of the Fpr1 agonist, fMLF (10 µM) and the Fpr-rs1 agonist, LXA4 (0.2 µM) on the release of ACTH from rat pituitary tissue *in vitro* evoked by forskolin (100 µM). Open columns = control; closed columns = forskolin. ** $P < 0.01$ vs. basal; †† $P < 0.001$ vs. forskolin alone; (ANOVA plus Duncan's multiple range test, $n=6$).

Figure 4.

Effects of the FPR antagonist, Boc2 (100 µM), in the presence and absence of dexamethasone (100 nM) on the release of ACTH from rat pituitary tissue *in vitro* evoked by forskolin (100 µM). Open columns = control; closed columns = forskolin. ** $P < 0.01$ vs. basal; † $P < 0.05$; †† $P < 0.001$ vs. forskolin alone; + $P < 0.05$ vs. corresponding Boc2-free group (ANOVA plus Duncan's multiple range test, $n=8$).

Figure 5.

Effects of dexamethasone (A; 100 nM) and ANXA1_{Ac2-26} (B; 6.8 μM) on the resting and forskolin-stimulated the release of ACTH from Fpr1 knockout (FPR KO) and WT pituitary tissue *in vitro*. Open columns = control; closed columns = forskolin (100 μM). **P* < 0.05 vs. basal; †*P* < 0.05, ††*P* < 0.01 vs. forskolin alone; +*P* < 0.05, ++*P* < 0.01 vs. corresponding WT control (ANOVA plus Duncan's multiple-range test, *n*=8).

Figure 6.

RT-PCR analysis of the expression of mRNAs of FPR family members in mouse pituitary tissue using 30 cycles (A) and 40 cycles (B) amplification and mouse hypothalamic tissue using 30 cycles (C) and 40 cycles (D) amplification. Note that Fpr1 mRNA was not detectable in mouse pituitary tissue at an amplification of 30 PCR cycles. Comparable data were obtained using the other primers shown in Table 1.

Figure 7.

Schematic diagram hypothesizing the role of FPR receptors in mediating the acute inhibitory actions on glucocorticoids on the regulation of ACTH release and, hence, the secretion of glucocorticoids. Annexin 1 (ANXA1, green spots) is normally abundant in the cytoplasm of folliculostellate (FS) cells, which are adjacent to corticotrophs. **A**) ACTH release is normally triggered by corticotrophin-releasing hormone (CRH) and arginine vasopressin (AVP), which synergistically act via specific receptors (CRH-R1 and V_{1b}-R) to increase intracellular 3'5' adenosine cyclic monophosphate (cAMP) and thereby activate protein kinase A (PKA). This triggers a sequence of events leading to Ca²⁺ entry via L-Ca²⁺ channels, vesicle fusion, and the release of ACTH by exocytosis. ACTH acts on the adrenal cortex to increase the synthesis and release of cortisol[b]. **B**) glucocorticoids (GCs) act via a glucocorticoid receptor (GR)-dependent mechanism in FS cells to activate a kinase cascade, which triggers the translocation of a serine-phosphorylate species of ANXA1 (ANXA1^{SPO4}) to the cell surface; the released protein acts on the adjacent corticotrophs via Fpr-rs1 and its associated heterotrimeric G-protein (G_i) to activate a signaling cascade, which triggers actin polymerization and thereby prevents vesicle fusion with the membrane and, hence, exocytosis. Note: Fpr-rs1 may also be a target for locally generated lipoxins, mitochondrially derived formylated peptides, and for microbial peptides, particularly formylated bacterial peptides and certain viral peptides. AC = adenylyl cyclase; PLC = phospholipase C; ATP = adenosine triphosphate; PIP₂ = phosphatidylinositol 4,5-bisphosphate; IP₃ = inositol trisphosphate; DAG = DAG; PKC = protein kinase A.

TABLE 1

Nucleotide sequences used to detect mRNAs of member of the FPR family in murine and rat pituitary tissue

Gene Name	Sequence	Manufacturer	Annealing Temperature	Expected Length
Mouse Fpr1 (553-570) forward	5' ACA GCC TGT ACT TTC GAC 3'	Sigma Genosys UK	62°C	563 bp
Mouse Fpr1 (1116-1097) reverse	5' CTG GAA GTT AGA GCC CGT TC 3' 5' GGC AAC TCT GTT GAG GAA AG 3'	Sigma Genosys UK	62°C	
Mouse Fpr-rs1 (538-557) forward	5' GGC TCT CGG	Sigma Genosys UK	62°C	422 bp
Mouse Fpr-rs1 (960-943) reverse	TAG ACG AGA 3' 5' GTC AAG ATC AAC AGA AGA AAC C 3'	Sigma Genosys UK	62°C	
Mouse Fpr-rs2 (676-697) forward	5' GGG CTC TCT CAA GAC TAT AAG G 3'	Sigma Genosys UK	62°C	297 bp
Mouse Fpr-rs2 (973-952) reverse	5' CCC CTG AGG AGC AAG TAA AAG TAT 3'	Sigma Genosys UK	62°C	421 bp
Mouse Fpr-rs6 (557-580) forward	5' CAG GGC TGA GTC CTC CCT TA 3' 5' CCT GAG GAG CAG GTA AAC ATG T 3'	Sigma Genosys UK	62°C	417 bp
Mouse Fpr-rs6 (978-959) reverse	5' GGG CTG AAT CCT CCC TCA 3' 5' AAG GTG AAG GTC GGA GTC AAC G 3'	Sigma Genosys UK	62°C	
Mouse Fpr-rs7 (559-580) forward	5' GGC AGA GAT GAT GAC CCT TTT GGC 3'	Sigma Genosys UK	62°C	
Mouse Fpr-rs7 (976-959) reverse				
GAPDH forward	5' GGC AGA GAT GAT GAC CCT TTT GGC 3'	Applied Biosystems UK	58°C	360 bp
GAPDH reverse		Applied Biosystems UK	58°C	