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Annexin 1, Glucocorticoids, and the Neuroendocrine–Immune Interface

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Abstract

Annexin 1 (ANXA1) was originally identified as a mediator of the anti-inflammatory actions of glucocorticoids (GCs) in the host defense system. Subsequent work confirmed and extended these findings and also showed that the protein fulfills a wider brief and serves as a signaling intermediate in a number of systems. ANXA1 thus contributes to the regulation of processes as diverse as cell migration, cell growth and differentiation, apoptosis, vesicle fusion, lipid metabolism, and cytokine expression. Here we consider the role of ANXA1 in the neuroendocrine system, particularly the hypothalamo-pituitary-adrenocortical (HPA) axis. Evidence is presented that ANXA1 plays a critical role in effecting the negative feedback effects of GCs on the release of corticotrophin (ACTH) and its hypothalamic-releasing hormones and that it is particularly pertinent to the early-onset actions of the steroids that are mediated via a nongenomic mechanism. The paracrine/juxtacrine mode of ANXA1 action is discussed in detail, with particular reference to the significance of the secondary processing of ANXA1, the processes that control the intracellular and transmembrane trafficking of the protein of the molecule and the mechanism of ANXA1 action on its target cells. In addition, the role of ANXA1 in the perinatal programming of the HPA axis is discussed.

Keywords

annexin 1; HPA axis; glucocorticoids; cytokines

INTRODUCTION

Annexin 1 (ANXA1, formerly known as lipocortin 1) is a 37-kDa member of the annexin superfamily of Ca^{2+} and phospholipid binding proteins. Members of this family show a high degree of structural homology, with each including four (or 8 in the case of ANXA6) repeated units of some 70 amino acids within the core and C-terminal regions; these units confer the Ca^{2+} and phospholipid binding properties of the proteins. The N-terminal region of each family member is, however, unique and is considered to confer the biological specificity of the individual proteins. The basic structure of ANXA1 is shown in Figure 1.

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Crystallography studies indicate that the four repeated sequences are arranged around a pore, giving the protein a "doughnut" appearance. The N-terminal domain is embedded within the pore at low Ca^{2+} concentrations, but elevations in [Ca^{2+}] expose this region and may thereby influence the biological activity of the protein.1 The gene encoding ANXA1 is well characterized in rodents (rat and mouse) and man2-4 (Fig. 1). Although the gene is multiexonic in structure, no spliced variants have been described to date. Sequence analysis has, however, revealed multiple potential sites for secondary processing, including tyrosine, serine, and threonine phosphorylation, acetylation, and lipidation. The protein may thus exist in multiple forms.

ANXA1 was first identified as a glucocorticoid (GC)-inducible protein in rat peritoneal macrophages and was heralded as a potential mediator of the powerful anti-inflammatory actions of these steroid hormones. Subsequent studies have confirmed that ANXA1 does indeed contribute to the anti-inflammatory actions of GCs. These studies have also revealed that the protein fulfills wider brief as in intra- and intercellular signaling molecule and that it is concerned with processes as diverse as cell growth and differentiation, apoptosis, vesicle fusion, endocytosis, exocytosis, and neuroprotection. Work in our laboratory has focused on the role of ANXA1 in the neuroendocrine system, particularly in relation to the negative feedback actions of GCs within the hypothalamo-pituitary-adrenocortical (HPA) axis.

EXPRESSION AND FUNCTION OF ANXA1 IN THE HPA AXIS

Localization

ANXA1 is expressed in abundance in the anterior pituitary gland, where it is localized specifically to the S100-positive folliculostellate cells.5,6 It is also found in lesser amounts in the hypothalamus, hippocampus, and other parts of the brain.7 Within the hypothalamus, ANXA1 is concentrated in the median eminence and the supraoptic and paraventricular nuclei.5 It is particularly prevalent in the ependymal cells lining the third ventricle and in activated glial cells but is not detectable in hypothalamic neurons.

Functional Studies

GCs act within the brain and pituitary gland to maintain the blood levels of GCs within appropriate limits by modulating the secretion of corticotrophin (ACTH) and its hypothalamic-releasing hormones, corticotrophin-releasing hormone (CRH) and arginine vasopressin (AVP). The actions of the steroids are complex and, in addition to acting at several different sites (e.g., pituitary gland, hypothalamus, hippocampus, ventral tegmental area), they also act via several different molecular mechanisms. GCs thus exert effects that are slow in onset (>2 h) and are characterized by suppression of the genes encoding ACTH, CRH, and AVP. In addition, they exert more immediate effects (onset 15–60 min) that inhibit the release of preformed hormone into the blood stream.

The early-onset actions of GCs within the HPA axis can be readily demonstrated in the rat and other species using *in vivo* and *in vitro* experimental systems. Thus, for example, the release of ACTH and corticosterone induced by stressors, such as restraint, endotoxin treatment, or surgical intervention, is readily suppressed by administration of GCs 15 min–1 h before the onset of stress. Similarly, *in vitro*, the stimulus-evoked release of ACTH and CRH/AVP from pituitary and hypothalamic tissue is inhibited by inclusion of GCs in the medium 15 min–1 h prior to addition of a secretagogue. Using these experimental models, we have identified an important role for ANXA1 in mediating the "early-onset" actions of GC in both the pituitary gland and the hypothalamus. We have thus shown that the inhibitory effects of GCs on the stress-induced release of ACTH and corticosterone *in vivo* are mimicked by intracerebroventricular (i.c.v.) injection of human recombinant ANXA1 (hrANXA1) or peptides derived from its N terminus and antagonized specifically by central

(i.c.v.) or peripheral subcutaneous (s.c.) administration of neutralizing anti-ANXA1 antisera (Fig. 2). Similarly *in vitro*, hrANXA1 mimics the inhibitory effects of GCs on the release of CRH and ACTH from rodent (rat or mouse) hypothalamic and pituitary tissue, respectively. Furthermore, the effects of the steroids are specifically reversed by neutralizing anti-ANXA1 antisera or antisense oligodeoxynucleotides (ODNs) directed specifically against ANXA1 mRNA.8-10 Interestingly, *in vivo,* ANXA1 appears to play a particularly important role in modulating the HPA responses to inflammatory stimuli.10,11 Similarly, at the hypothalamic level, ANXA1 is particularly effective in suppressing the CRH responses to proinflammatory cytokines *in vitro*.11

MECHANISM OF ANNEXIN 1 ACTION WITHIN THE HPA AXIS

The Paracrine/Juxtacrine Hypothesis

Studies involving cell fractionation techniques and electron microscopic immunohistochemistry have shown that ANXA1 is distributed between three cellular compartments, namely, the cytoplasm, within the membranes of cell organelles, and in association with the plasma membrane, where a proportion of the protein is localized to the outer cell surface.6,7 Early work in our laboratory revealed that GCs regulate both the synthesis and the cellular disposition of ANXA1 in the anterior pituitary gland and the hypothalamus.9,11 GCs thus induce *de novo* ANXA1 synthesis in these tissues and cause the translocation of the protein from the cytoplasm to the outer cell membrane, where it is retained by a Ca^{2+}-dependent mechanism. The transfer of cytoplasmic ANXA1 to the cell surface is apparent within 15 min of GC contact both *in vivo* and *in vitro*. By contrast, the induction of ANXA1 synthesis is slow in onset (>2 h) and appears to serve primarily as a mechanism for replenishing the diminished cytoplasmic pool of the protein.7

The findings that ANXA1 is not expressed by CRH/AVP neurons or corticotrophs, but is present in the nonendocrine cells adjacent to them, together with evidence that the protein is promptly translocated to the cell surface by GCs, led us to propose that ANXA1 acts via paracrine/juxtacrine mechanism to mediate the inhibitory effects of the steroids on CRH/AVP and ACTH release. Several lines of evidence support this hypothesis. Firstly, specific, high-affinity (Kd = 14 ± 3 nM), proteinaceous ANXA1 binding sites are expressed on the cell surface of the corticotrophs and other endocrine cells in the anterior pituitary gland.12 Secondly, enzymatic destruction of these sites ablates ANXA1 binding and the antisecretory effects of both GCs and ANXA1 on ACTH secretion in primary cultures of rat pituitary tissue, suggesting that these sites play an important role in mediating ANXA1 actions.12 Thirdly, immunogold labeling has revealed that ANXA1 release occurs at focal points on the FS cell membranes, particularly at loci where the stellar projections of the cells make close contact with the endocrine cells13 (Fig. 3). Finally, drugs that prevent the GC-induced translocation of ANXA1 to the cell membrane (e.g., PKC inhibitors, ATP-sensitive K^+ channel openers) overcome the acute inhibitory effects of GCs on ACTH secretion in *in vitro* models.14,15

Confirmation that ANXA1 serves as cell–cell mediator of GC action in the pituitary gland was provided by studies on a co-culture system comprising two cell lines, viz., murine corticotroph (AtT20, clone D1) and murine FS (TtTGF). TtT/GF cells express ANXA1 in abundance and readily translocate the protein to the cell surface in response to a GC challenge.16 By contrast, while specific ANXA1 binding sites are readily detected on the cell surface of the AtT20 D1 cells by FACS analysis, the cell line does not express ANXA1 mRNA or protein as detected by RT-PCR and Western blot analysis. These two cell lines thus mirror the pattern of expression of ANXA1 and ANXA1 binding observed *in vivo* and, thus, provide a useful model for exploration of ANXA1 biology. When cultured alone, the AtT20 D1 cells are unresponsive to GCs but respond readily to ANXA1 and peptides

derived from it. The CRH-induced release of ACTH from the cells is thus unaffected by the inclusion of dexamethasone in the incubation medium, but is readily blocked by ANXA1 and $ANXA1_{Ac2–26}$. However, when co-cultured with TtT/GF cells, the AtT20 D1 cells respond to dexamethasone with a marked reduction in CRH-induced ACTH release; furthermore, the magnitude of the response to dexamethasone is proportional to the ratio of TtT/GF to AtT20 D1 cells in culture (Fig. 4). The importance of ANXA1 in mediating this action of dexamethasone was confirmed by antisense studies that showed clearly that the effects of the steroid were ablated by antisense ODNs directed against ANXA1 mRNA but not by corresponding sense- or antisense sequences.16

Translocation of ANXA1 to the Cell Surface

The amino acid sequence of ANXA1 does not include a signal sequence. Consequently, the protein is not packaged into vesicles by the Golgi system for release by exocytosis, and its translocation to the cell membrane is unaffected by drugs that interfere with various steps within the exocytotic pathway (e.g., nocodazole, brefeldin A).7 Early studies indicated that the association of ANXA1 with membranes is strongly influenced by the phosphorylation status of the molecule.17 Accordingly, we examined the potential role of phosphorylation in the GC-induced translocation of ANXA1 to cell membrane. Our initial studies indicated that cell surface ANXA1 is phosphorylated on both serine and tyrosine residues.14,18 However, while blockade of PKC-dependent serine phosphorylation prevents the GC-induced translocation of ANXA1 to the cell surface of primary FS cells,14 inhibition of tyrosine phosphorylation does not.18 Together these data suggest that serine but not tyrosine phosphorylation is critical to the GC-induced transfer of ANXA1 across the cell membrane. Further evidence to support a role for serine phosphorylation emerged from studies on a human FS cell line (PDFS). Work on this line also demonstrated that the actions of GCs are mediated via the glucocorticoid receptor but that downstream signaling is effected by a nongenomic mechanism that uses PI3 kinase and MAP kinase as well as PKC.19 The ANXA1 sequence includes multiple serine phosphorylation sites; and we are therefore exploiting site-directed mutagenesis and green fluorescent protein (GFP) tagging to determine the relative importance of each. Our initial data suggest that serine-27 is important in this regard, but other sites, particularly serine-5, may also have a significant role. In addition, they have identified a potential role for protein lipidation in the signaling cascade.20

The mechanism by which phosphorylated ANXA1 traverses the membrane is poorly understood. Double-labeling immunohistochemistry has shown that ANXA1 co-localizes with an ATP binding cassette (ABC) transporter protein (ABCA1) at focal points in the membrane of primary and transformed (TtT/GF) FS cells, thus raising the possibility that this protein may be important in this regard.21 This hypothesis was supported by the evidence that glyburide, which blocks ABCA1, inhibits not only the GC-induced exportation of ANXA1 in FS cells and primary pituitary tissue, but also the ANXA1-dependent inhibitory effects of dexamethasone on ACTH release from pituitary tissue.21 However, doubts regarding the role of ABCA1 have emerged, as Tangiers lymphocytes, which lack the ability to traffic ABCA1 to the cell membrane, readily export ANXA1 in response to a steroid challenge. This unexpected finding could be explained by the development of a compensatory mechanism. However, glyburide is not specific to ABCA1, but also opens ATP-sensitive K^+ channels (K^+_{ATP} channels). These channels are expressed by FS cells. Moreover, the K^+_{ATP} channel blockers, chromokalim and minoxidil, induce the translocation of ANXA1 to the cell surface of TtT/GF cells in a concentration-dependent manner; furthermore, their effects are blocked by glyburide and other K^+_{ATP} channel openers. Thus, while the role of ABCA1 is uncertain, it seems likely that K^+_{ATP} channels contribute to the signaling mechanism involved in the membrane translocation of ANXA1.

Actions of ANXA1 on Endocrine Cells

As discussed above, there is good evidence that ANXA1 acts via specific membrane-bound receptors on the corticotrophs and other pituitary endocrine cells to suppress pituitary hormone release.12 The nature of these receptors is ill-defined, but data from other systems have raised the possibility that formyl peptide receptors (FPRs) may be important in this regard.22 In humans, three members of this G-protein-coupled family of receptors have been characterized, namely, FPR, FPR-L1, and FPR-L2. Furthermore, ANXA1 and certain peptides derived from it have been shown to bind to both FPR and FPR-L1. However, in rodents the family is more extensive with eight or possibly nine family members. The majority are poorly characterized, but Fpr1 and Fpr-rs1/Fpr-rs2 are mooted to be the murine equivalents of the human FPR and FPR-L1. We have recently shown that Fpr1 is not expressed in the mouse or rat pituitary gland and that Fpr1 gene deletion does not affect the capacity of ANXA1 or dexamethasone to suppress ACTH release from murine pituitary tissue. Other members of the FPR family are, however, found in the murine anterior pituitary gland. Furthermore, the inhibitory effects of dexamethasone and ANXA1 on ACTH release are mimicked by lipoxin A4, a lipid mediator purported to be an endogenous ligand of FPR-L1, and by high concentrations of the bacterial peptide, formyl-methione-leucine-proline (fMLF), which shows low affinity for FPR-L1. These findings, together with evidence that the effects of GCs, ANXA1, fMLF, and lipoxin A4 on ACTH release are blocked by N-Boc-MLP, an analogue of fMLP, which shows antagonist activity at both FPR and FPR-L1, supports the premise that a member of the FPR family contributes to the regulatory actions of ANXA1 on ACTH release. The signaling mechanism downstream of the receptor is largely unexplored. However, other data suggest that ANXA1 disrupts signaling at a point distal to the formation of cyclic AMP and the entry of Ca^{2+} and that, while the protein readily suppresses adenylyl cyclase-dependent secretagogue signaling, it does not affect phospholipase C-driven exocytosis.8-10,14,23

The sequences within the ANXA1 molecule that are required for the inhibition of peptide release have been explored using peptide analogues.14 Removal of the third and fourth repeat units of the core ($ANXA1_{1–188}$) has no effect on either the potency or the efficacy of the protein in the pituitary gland. However, additional loss of the second repeat and much of the first repeat ($ANXA1_{1–50}$) results in a significant loss of potency but not efficacy. $ANXA1_{Ac2–26}$ shows a marked reduction in both potency and efficacy, and shorter N-terminal sequences (e.g., $ANXA1_{Ac–12}$, $ANXA1_{Ac13–26}$) are without effect.18 These data support the premise that the N-terminal plays an essential role in the manifestation of ANXA1 action, as also does our finding that neutralizing antisera directed specifically against the N-terminal domain attenuate the inhibitory effects of GCs on ACTH release *in vivo* (Fig. 2). However, they also reveal marked differences in the activity profile and potency ratios of the peptides studied in this and other systems (e.g., models of inflammation; see Fig. 5), where, for example, $ANXA1_{1–188}$ is less potent than the parent molecule ($ANXA1_{1–346}$ and $ANXA1_{Ac2–26}$ displays full efficacy.24 Taken together, these data raise the possibility that two or more receptor subtypes mediate the actions of ANXA1 in the body, a view that is consistent with a role for two or more members of the FPR family.

EARLY LIFE PROGRAMMING OF THE ANXA1 SYSTEM

The ontogeny of ANXA1 expression in the developing fetus/neonate has not been studied in detail. However, striking tissue-specific changes in ANXA1 expression occur in the neonate at the time of the stress hyporesponsive period (SHRP). In particular, ANXA1 expression in the anterior pituitary gland and hippocampus is raised (vs. adult) while thymic ANXA1 is reduced.25 Complementary functional experiments suggest that the upregulation of ANXA1 in the neuroendocrine system may be an important factor in the maintenance of the

quiescent state of the HPA axis during this period. ANXA1 may thus serve to protect the developing organism from the potentially harmful effects of overexposure to GCs at this time.25 The regulatory effects of ANXA1 on AVP from the hypothalamus appear to be particularly important in this regard as, in addition to facilitating the inhibitory actions of GCs in the release of CRH/AVP and ACTH from the hypothalamus and pituitary gland, respectively, ANXA1 exerts a tonic inhibitory effect on AVP release at this stage and thereby quenches that stimulatory effects of interleukin IL-1α, IL-1β, and IL-6 on the release of the peptide.25

Interestingly, administration of very low doses of dexamethasone to dams via the drinking water in late gestation (E16–19) or post partum (day 1–7) causes tissue-specific changes in ANXA1 expression in the brain and pituitary gland of the offspring that persist into adulthood.26 Furthermore, ANXA1-dependent GC feedback is blunted in animals exposed to exogenous GCs in the perinatal period, apparently because the GC-induced translocation of ANXA1 to cell surface is compromised.26 These and other (unpublished) data raise the possibility that ANXA1 contributes to the disturbances in HPA function and associated pathologies that are evident in adults rats treated with GCs at critical stages of development.

CONCLUSIONS

Since its discovery in 1979, ANXA1 has been shown to play a diverse role as a signaling molecule in a number of physiological and pathophysiological systems, including the neuroendocrine system as discussed in this article. Our data suggest that ANXA1 acts via a paracrine/juxtacrine mechanism to suppress the release of ACTH and its hypothalamic-releasing hormones (Fig. 6). New technologies now pave the way for important new insights to the mechanisms which regulate the secondary processing and trafficking of ANXA1, to the physiological significance of these processes, and to the molecular mechanisms of ANXA1 action. The knowledge that emerges from these studies should provide new insight into the role of this protein in health and disease and may ultimately lead to the therapeutic exploitation of ANXA1-mimietics.

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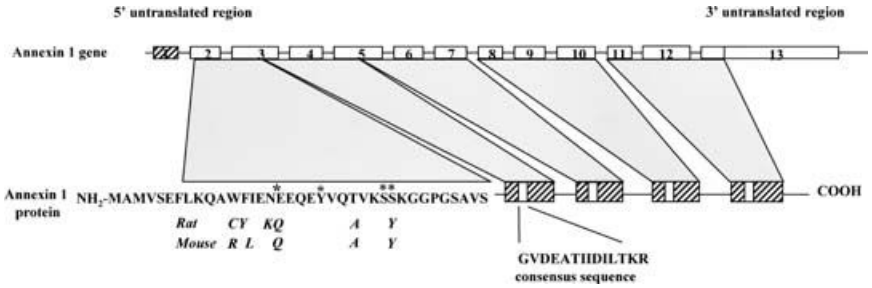

FIGURE 1.

Schematic diagram to show the structure of the human annexin 1 gene and protein. Note that the N-terminal includes potential sites for phosphorylation (*) and that each of the four repeats in the core domain includes a 17–amino acid consensus sequence, which is critical to Ca^{2+} binding. Differences in the N-terminal amino acid sequence of the rat and mouse proteins are indicated in *italics*. (Reprinted by permission from *Endocrinology8*).

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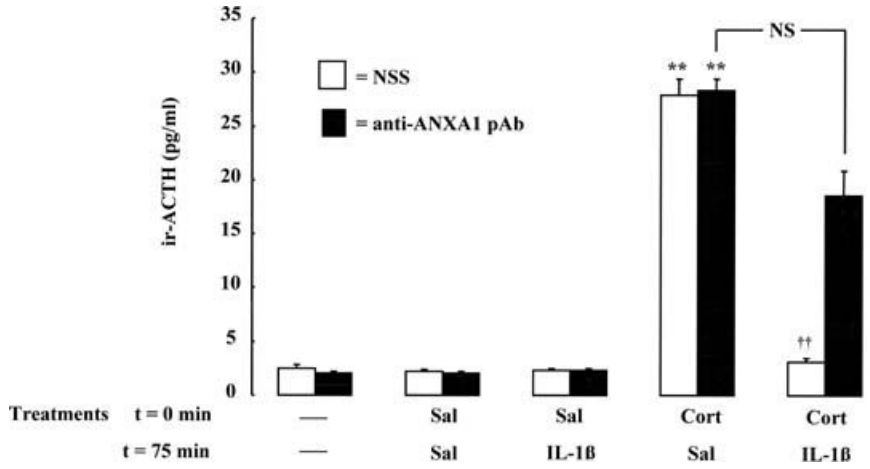

FIGURE 2.

Reversal of the inhibitory effects of corticosterone on the IL-1β-stimulated release of ACTH in adult male rats by administration of an antiannexin 1 polyclonal antiserum raised in sheep against $ANXA1_{Ac2–26}$. Corticosterone 500 μg/kg, i.p in a volume of 1 mL/kg; IL-1β 10 ng/rat in a volume of 3 μL, i.c.v. Controls received equivalent volumes of the sterile saline (Sal) vehicle. Antiannexin 1 antiserum (anti-ANXA1 pAb) 1mL/kg, s.c. or nonimmune sheep serum (NSS, control) 1mL/kg, s.c. was administered 15 min before the steroid. Values represent mean ± SEM (n = 6–7). ** $P < 0.01$ versus Sal–Sal treated control; †† $P < 0.01$ versus Sal–IL-1-β treated group; NS = not significant ($P > 0.05$). (ANOVA plus Scheffé's test).

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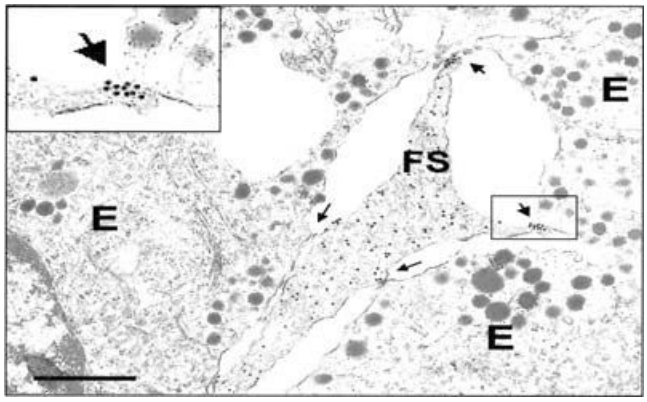

FIGURE 3.
Electron micrograph showing immunogold detection of annexin 1 in a folliculostellate cell adjacent to three endocrine (E) cells in freeze-substituted mouse anterior pituitary tissue. Gold particles (15 nm) are scattered over the cytoplasm and adjacent to the plasma membrane of the cell; they are also localized on the FS cell surface at the ends of the processes contacting endocrine cells (see enlarged *inset*). *Arrows* indicate intercellular junctions. *Scale bar:* 1 μm. (Reprinted by permission from *Endocrinology13*).

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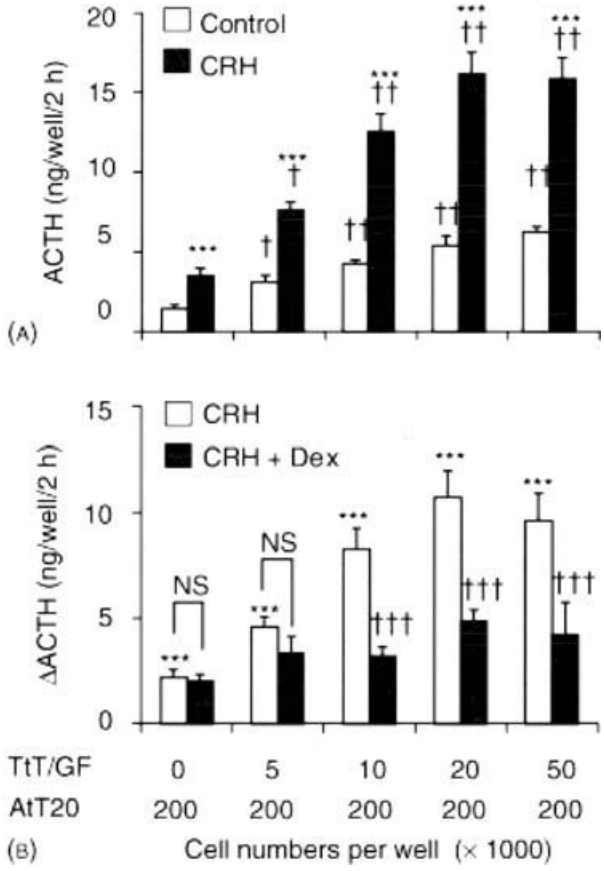

FIGURE 4.

Effects of corticotrophin-releasing hormone (CRH 1 μm) and dexamethasone (Dex 100 nm) on the secretion of adrenocorticotrophic hormone (ACTH) by AtT20 D1 cells (200,000 per well) cultured alone and in the presence of increasing numbers of TtT/GF cells (5,000–50,000 cells per well). (**A**) basal and CRH-stimulated release of ACTH. ***$P < 0.001$ basal versus CRH. †$P < 0.05$, ††$P < 0.01$ co-cultures versus AtT20 D1 cells alone. (**B**) The effects of Dex on CRH-stimulated ACTH release; Dex had no effect on basal ACTH release (data not shown). ***$P < 0.001$ Basal versus CRH. †††$P < 0.001$ CRH + Dex versus corresponding group treated with CRH alone. Data shown are mean ± SEM of six wells per group. Statistical analysis was carried out by ANOVA and Tukey's *post hoc* test. The data shown are representative of three separate experiments. (Reprinted by permission from the *Journal of Neuroendocrinology16*).

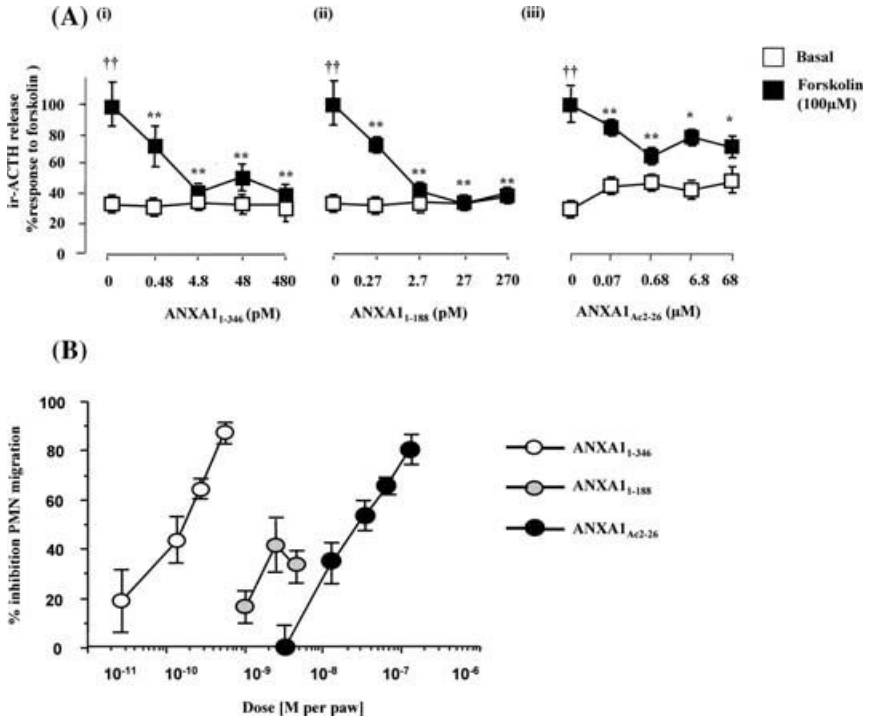

FIGURE 5.

Comparison of the biological activity of full-length human recombinant annexin 1 (hrANXA1$_{1–346}$), a truncated protein (ANXA1$_{1–188}$), and an N-terminal peptide (ANXA1$_{Ac2–26}$) on (*a*) pituitary function and (*b*) in a model of inflammation. (**A**) demonstrates the inhibitory effects of graded concentrations of (i) ANXA1$_{1–346}$, (ii) ANXA1$_{1–188}$, and (iii) ANXA1$_{Ac2–26}$ on the release of ir-ACTH from rat anterior pituitary segments in *in vitro* data. Mean ± SEM ($n = 6$) are expressed as percentage of the secretory response to forskolin alone (100 μM). *$P < 0.05$, **$P < 0.01$ versus forskolin alone group (ANOVA and Duncan's multiple range test). (**B**) demonstrates the effects of the peptides *in vivo* on the IL-1 β-induced migration of neutrophils into a mouse air pouch migration (elicited by *in vivo* injection of IL-1-β into a small air pouch in the mouse). ANXA1 protein/peptides were co-injected with IL-1-β; the data are expressed as the mean ± SEM, *n*-4–10. Note (*a*) that in the pituitary gland, ANXA1$_{1–364}$ and ANXA1$_{1–188}$ are roughly equipotent, whereas in the air pouch model ANXA1$_{1–346}$ is approximately two orders of magnitude more potent than ANXA1$_{1–188}$, (*b*) that ANXA1$_{Ac2–26}$ shows the full efficacy of the parent protein in the air pouch model but not in the pituitary gland, where at best it produces a 60% inhibition of peptide release and (*c*) that while ANXA1$_{Ac2–26}$ is less potent than the parent protein in both models, the potency difference is considerably greater in the pituitary gland. (Reprinted by permission from *Trends in Endocrinology and Metabolism24*).

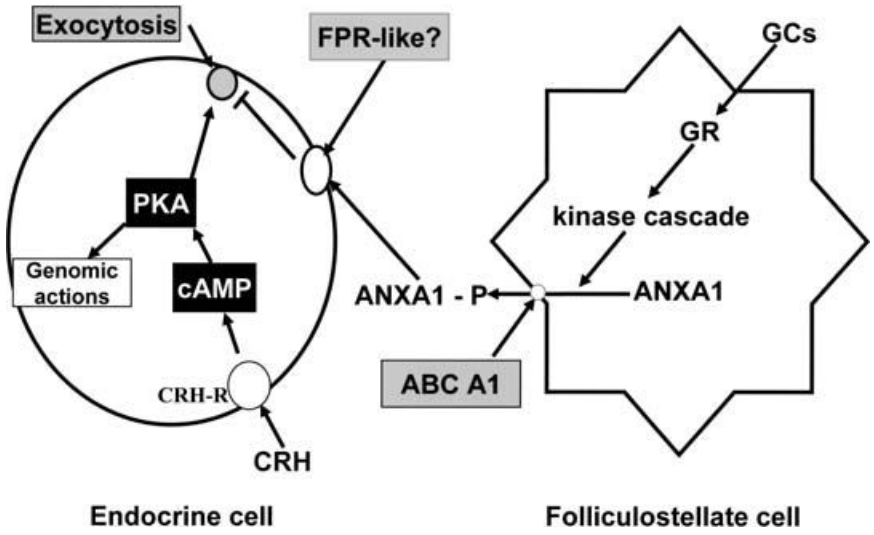

FIGURE 6.
Schematic diagram illustrating the proposed mechanism by which ANXA1 produced and stored in folliculostellate cells acts as paracrine/juxtacrine mediator of the early inhibitory effects of GCs on the release of ACTH from the corticotrophs. CRH = corticotrophin-releasing hormone; CRH-R = corticotrophin-releasing hormone receptor; PKA = protein kinase A; GC = glucocorticoid; GR = glucocorticoid receptor; ANXA1 = annexin 1; ANXA1–P = serine-phosphorylated ANXA1; ABC A1–ATP binding cassette protein A1, the putative ANXA1 transporter; FPR-like = formyl peptide receptor like, the putative ANXA1 receptor. (Reprinted by permission from *Trends in Endocrinology and Metabolism24*).

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