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Expression and Externalization of Annexin 1 in the Adrenal Gland: Structure and Function of the Adrenal Gland in Annexin 1-Null Mutant Mice

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Annexin 1 (ANXA1) is a member of the annexin family of phospholipid- and calcium-binding proteins with a well demonstrated role in early delayed (30 min to 3 h) inhibitory feedback of glucocorticoids in the hypothalamus and pituitary gland. This study used adrenal gland tissue from ANXA1-null transgenic mice, in which a β-galactosidase (β-Gal) reporter gene was controlled by the ANXA1 promoter, and wild-type control mice to explore the potential role of ANXA1 in adrenal function. RT-PCR and Western blotting revealed strong expression of ANXA1 mRNA and protein in the adrenal gland. Immunofluorescence labeling of ANXA1 in wild-type and β-Gal expression in ANXA1-null adrenals localized intense staining in the outer perimeter cell layers. Immunogold electron microscopy identified cytoplasmic and nuclear ANXA1 labeling in outer cortical cells and capsular cells. Exposure of adrenal segments *in vitro* to dexamethasone (0.1 μM, 3 h) caused an increase in the amount of ANXA1 in the intracellular compartment and attached to the surface of the cells. The N-terminal peptide $ANXA1_{Ac2–26}$ inhibited corticosterone release. Corticosterone release was significantly greater from ANXA1-null adrenal cells compared with wild type in response to ACTH (10 pM to 5 nM). In contrast, basal and ACTH-stimulated aldosterone release from ANXA1-null adrenal cells was not different from wild type. Morphometry studies demonstrated that ANXA1 null adrenal glands were smaller than wild-type, and the cortical/medullary area ratio was significantly reduced. These results suggest ANXA1 is a regulator of adrenocortical size and corticosterone secretion. (*Endocrinology* 148: 1030–1038, 2007)

ANNEXIN 1 (ANXA1) IS a glucocorticoid-regulated protein that has been implicated in the regulation of phagocytosis, cell signaling, growth, and proliferation and to be a mediator of glucocorticoid action in inflammation (1). ANXA1 also contributes to the regulatory actions of glucocorticoids in the control of the hypothalamo-pituitary-adrenal axis (2). Functional studies using neutralizing antisera, antisense oligodeoxynucleotides, and various ANXA1-related N-terminal peptides, such as peptide $ANXA1_{Ac2–26}$, have identified a key role for ANXA1 in effecting acute, nontranscriptional inhibitory actions of the glucocorticoids on the release of ACTH (3–5). Similarly, at the hypothalamic level, ANXA1 peptides mimic the ability of dexamethasone to suppress the release of CRH-41 evoked by cytokines (6, 7). Glucocorticoids in several tissues induce externalization of ANXA1 from the cytoplasm to the outer cell surface where it is retained by a Ca^{2+}-dependent mechanism (3, 8). Externalization of ANXA1 is stimulated by glucocorticoid treatment in a time- and concentration-dependent manner (9, 10). The steroids also induce *de novo* ANXA1 synthesis to replenish the intracellular stores of the protein (5). Functional and binding studies suggest that the glucocorticoid-induced translocation of ANXA1 mediated by an ATP-binding cassette transporter (11, 12) is an important mechanism that enables the protein to access binding sites on the surface of cells and thereby exert their actions (13). ANXA1-binding sites have been identified on the surface of pituitary cells, monocytes, and neutrophils and may be related to the formyl peptide-related receptor family, which has been implicated in ANXA1 signaling (13–15). Recent studies have shown that ANXA1-null transgenic mice exhibit a prolonged and exacerbated inflammatory response and are partially resistant to the antiinflammatory properties of glucocorticoids (16–18).

Despite the importance of ANXA1 in mediating the regulatory effects of glucocorticoids on hypothalamic and pituitary function, it is unknown whether ANXA1 regulates adrenal gland function directly. The adrenal gland itself regulates a number of essential physiological functions through the production of glucocorticoids, mineralocorticoids, and catecholamines. The glucocorticoid-producing zona fasciculata of the adrenal cortex is dependent upon ACTH and other proopiomelanocortin-derived peptides produced by pituitary corticotropes to maintain normal structure and function (19, 20). Glucocorticoids in turn induce atrophy of the adrenal cortex (21, 22) and reduce the proliferation of zona fasciculata cells (23). In the present study, we have used histological, molecular, and functional approaches to explore the effects of ANXA1 on adrenal gland function using the ANXA1-null transgenic mouse as an experimental model.

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Abbreviations: ANXA1, Annexin 1; FCS, fetal calf serum; β-Gal, β-galactosidase; HPF, high-power field.

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Materials and Methods

Animals

ANXA1-null mice were generated by targeting of the ANXA1 gene in embryonic stem cells as described (16). Gene targeting was performed by homologous recombination in embryonic stem cells derived from strain 129 Agouti mice. Correctly targeted embryonic stem cells were then injected into blastocysts from C57 Black females. The resulting chimeric males were test-bred with C57 Black females to produce (129 × C57) F1 offspring, which were then mated together to produce F2 homozygous knockout animals. A colony of these mice was established in the Central Biomedical Services, Imperial College London, Hammersmith Campus, by rederivation. The targeting construct disrupted expression of ANXA1 and placed a β-galactosidase (β-Gal) reporter gene in frame as a fusion gene with the first three amino acids of ANXA1. Female wild-type littermate control and ANXA1-null mice (20–25 g body weight) were maintained on a standard chow pellet diet with tap water *ad libitum*. Animals were housed in groups of five per cage in a quiet room with controlled lighting (lights on 0800–2000 h) and temperature maintained at 21–22 C. All experiments were started between 0800 and 0900 h to avoid changes associated with the circadian rhythm. The *Principles of Laboratory Animal Care* (National Institutes of Health publication no. 85-23) were followed, and animal work was carried out under license in accordance with the *UK Guidance on the Operation of Animals, Scientific Procedures Act 1986*.

For Western blot and real-time PCR studies, mice were terminally anesthetized by ip injection of 3 mg sodium pentobarbital (Sagatal, Rhone Merieux, France) and perfused through the heart with heparinized saline (0.9% NaCl and 10 U/ml heparin). Perfusion with saline ensured that ANXA1-rich leukocytes were removed before assay. For real-time PCR, tissues (anterior pituitary, spleen, brain, thymus, and adrenal glands) were removed for immediate extraction of RNA. For Western blotting, tissues were frozen immediately on dry ice and stored at −80 C before analysis. For immunofluorescence, β-Gal histochemistry, and electron microscopy, mice were terminally anesthetized as above and perfused through the heart with heparinized saline (0.9% NaCl and 10 U/ml heparin) followed by 4% paraformaldehyde in PBS. The adrenal glands were removed and immersed in the same fixative for 3 h at 4 C. For preparation of isolated adrenal cells, adrenal glands were collected immediately into ice-cold MEM (Life Technologies, Inc., Paisley, UK) after cervical dislocation. For histological analysis, adrenal glands were collected immediately into Bouin's solution and fixed for 24 h.

RT-PCR for ANXA1, MC2R, and GR mRNA

Total RNA was isolated from adrenals and pituitary from wild-type and ANXA1-null mice using the RNeasy mini kit (QIAGEN, Crawley, UK) following the manufacturer's instructions, eluted in 40 μl water, and treated with DNase (DNA-free; Ambion, Witney, UK) to remove traces of genomic DNA contamination. From each sample, 3.5 μg of total RNA was subsequently reverse transcribed using 1 μl Maloney murine leukemia virus reverse transcriptase (Clontech, Oxford, UK), 2 μl 10 mM dNTP, and 2 μl 100 mM dithiothreitol in a total volume of 20 μl. In all experiments, a negative control was also run omitting the RT enzymes in the reaction. PCR was carried out using the following primers: ANXA1, forward primer 5′-ATGGCAATGGTATCAGAATTCCTCAAGC-3′ and reverse primer 5′-GATGTCTAGTTTCCACCACACAGAGCC-3′ (expected size, 1062 bp); GR, forward primer 5′-CATGTTAGGTGGGCGCCAAGTG-3′ and reverse primer 5′-CCCTCTAGAGACCACATGTAGTGCG-3′ (expected size, 820 bp); and MC2R, forward primer 5′-CCGCACCATCATCACCCTAACAATT-3′ and reverse primer 5′-TGCCATTGACCTGGAAGAGAGACATG-3′ (expected size, 500 bp). Each reaction also contained primers for GAPDH that served as an internal control: forward primer 5′-ACTCACGGCAAATTCAACGGCACAG-3′ and reverse primer 5′-TGGTCATGAGCCCTTCCACAATGCC-3′ (expected size, 376 bp). All primers were synthesized by Sigma-Genosys (Hinxton, UK). One microliter of cDNA was used as a template in the PCR (total volume 50 μl). PCR conditions were optimized to an annealing temperature of 68 C and 40 cycles (Expand High Fidelity PCR system; Roche, Hemel Hempstead, UK). PCR products were separated on a 1% agarose gel stained with ethidium bromide, photographed, and analyzed by scanning densitometry (TINA software version 2.10; Raytest Isotopenmessgeraete GmbH, Straubenhardt, Germany).

Real-time PCR. Relative expression of adrenal ANXA1 mRNA to spleen, pituitary, brain, and thymus was monitored by real-time PCR. Total RNA was isolated using TriReagent and treated with DNase (DNA-free; both from Ambion) to remove traces of genomic DNA contamination. Total RNA, 5 μg from each sample, was subsequently reverse transcribed using 1 μl Moloney murine leukemia virus reverse transcriptase (Clontech), 2 μl 10 mM dNTP, and 2 μl 100 mM dithiothreitol in a total volume of 20 μl. ANXA1 expression was then detected and quantified by real-time PCR using 12.5 μl Absolute QPCR mix (ABgene, Epsom, UK), 0.4 μM each of ANXA1 forward primer and ANXA1 reverse primer and probe mix (Predeveloped TaqMan Assay; Applied Biosystems, Warrington, UK) in a total volume of 25 μl. The QPCR assay was performed in an ABI PRISM 7000 (Applied Biosystems) and cycled as follows: initial enzyme activation at 95 C for 15 min and then 40 cycles of 95 C for 15 sec and 60 C for 1 min. The assays were normalized with 18S RNA primers and probes (Applied Biosystems) and the results expressed as the amount of ANXA1 mRNA/18S RNA.

Detection of ANXA1 by Western blot analysis

Cell surface and intracellular ANXA1 was extracted from adrenal tissue as described previously (3, 10, 11). Briefly, cell surface ANXA1 was removed from the outer cell membranes by washing the tissue or cells gently for 2 min in a solution containing 1 mM EDTA in PBS, which, by chelating Ca^{2+}, releases ANXA1 into the medium from Ca^{2+}-dependent cell surface binding sites. Intracellular ANXA1 was extracted from the remaining adrenal tissue by sonication (25 Hz for 20 sec) (Soniprep 150; MSE, London, UK) on ice in PBS containing EDTA (10 mM), Triton X-100 (1% vol/vol) (Sigma Chemical Co., Poole, UK), and phenylmethylsulfonyl fluoride (1 mM) (Sigma). The total protein content of each sample was determined by use of a BCA protein assay reagent (Pierce Chemical Co., Chester, UK). The extracted proteins were separated by SDS-PAGE [20 μg/channel in a volume of 20 μl by use of a midget gel Hoeffer electrophoresis system and power pack (LKB, Milton Keynes, UK)] and transferred electrophoretically to nitrocellulose paper (Bio-Rad Laboratories Inc. Ltd., Hemel Hempstead, UK). ANXA1 was detected by overnight incubation (4 C) with a well-characterized (8) sheep anti-ANXA1 polyclonal antibody (diluted 1:1000) followed by antisheep IgG conjugated to horseradish peroxidase (diluted 1:5000) (Sigma). The housekeeping protein β-actin was also detected with a monoclonal anti-β-actin antibody, clone AC-15 (1:5000) and peroxidase-conjugated antimouse secondary antibody (both from Sigma). Immunoreactive bands were detected by chemiluminescence reagents and exposed to Hyperfilm (both from Pierce). The blots were scanned using a flatbed scanner (HP Scanjet 5200 with Adobe Photodeluxe Business Edition version 1.1) and the band intensity analyzed using the TINA software program (TINA version 2.10; Raytest Isotopenmessgeraete GmbH, Germany). Intensity values were normalized relative to control values.

Static incubation of adrenal glands in vitro: effect of dexamethasone treatment on ANXA1 distribution

Adrenal glands were cut into four segments. The segments were distributed randomly (one segment per well) in the wells of 24-well tissue culture plates (Costar, Cambridge, MA) and incubated at 37 C for 3 h in either 1 ml incubation medium (MEM supplemented with 5% fetal calf serum (FCS), 0.5% penicillin/streptomycin, and 0.5% glutamine) or incubation medium containing 0.1 μM dexamethasone under a humidified atmosphere saturated with 95% O_2/5% CO_2. The medium was changed at 1 and 1.5 h. Adrenal segments were extracted for subsequent Western blot analysis of surface and intracellular ANXA1 (see *Detection of ANXA1 by Western blot analysis* above).

Fluorescence microscopy

For localization of ANXA1 by immunofluorescence, wild-type adrenal glands were immunolabeled with a polyclonal ANXA1 antibody, and ANXA1-null adrenal glands were immunolabeled with a monoclonal anti-β-Gal antibody. Perfusion-fixed, sucrose-cryoprotected tissue was embedded in Tissue Tek OCT (Miles, Elkhart, IN) on dry ice.

The 10-μm cryostat sections were prepared onto gelatin-coated glass slides. Sections were rinsed in PBS, blocked with 10% FCS in PBS at room temperature for 1 h, and incubated for 2 h with either sheep antihuman ANXA1 polyclonal antibody (1:1000) or mouse anti-β-Gal (1:200) (Sigma) in 10% FCS in PBS. Immunoreacted sections were washed with PBS and then incubated for 1 h at room temperature with either a fluorescein-conjugated goat antisheep IgG (Vector Laboratories Inc., Burlingame, CA) for ANXA1 visualization or a fluorescein-conjugated donkey antimouse IgG (Vector) for β-Gal localization. The slides were mounted in Vectashield mounting medium (Vector) and examined using a TCS confocal microscope (Leica Corp. Microsystems, Wetzlar GmbH, Germany). Nonspecific staining and background were assessed by substitution of either nonimmune sheep sera or ANXA1 antisera preincubated with a 100-fold excess of recombinant human ANXA1 for primary antisera. For β-Gal staining, labeling of wild-type sections was performed as a negative control.

X-Gal histochemistry

Perfusion-fixed adrenal glands were cut into sections (100 μm) with a Vibratome (Camden Instruments, Sileby, UK) and incubated for 2.5 h at room temperature in X-Gal substrate solution (1 mg/ml) containing 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 2 mM $MgCl_2$, 0.01% sodium deoxycholate, and 0.02% Nonidet P-40 (pH 7.2). The pH conditions had been carefully optimized to minimize nonspecific labeling. When the color development was complete, the tissue was rinsed with cold PBS and the reaction was stopped by transferring the tissue to 4% paraformaldehyde in PBS. Sections were counterstained with carminic acid (Sigma), mounted in Hydromount (BDH Merck, Poole, UK) and viewed with an Olympus BH2 microscope (Olympus UK Ltd., Middlesex, UK).

Immunoelectron microscopy

Perfusion-fixed male wild-type and ANXA1-null adrenal glands, postfixed in freshly prepared 4% paraformaldehyde in PBS for 4 h at 4 C, were washed briefly in PBS and transferred to a solution of 2.3 M sucrose in PBS overnight. The cryoprotected tissue was cut into 300-μm-thick slices with a Vibratome (Camden Instruments), slam-frozen (Reichert MM80E; Leica, Milton Keynes, UK), freeze-substituted at −80 C in methanol for 48 h, and embedded at −20 C in LR Gold acrylic resin (London Resin Co. Ltd., Reading, UK) in a Reichert freeze-substitution system. Ultrathin sections were prepared by use of a Reichert Ultracut S ultratome, mounted on 200-mesh nickel grids, incubated for 2 h with the sheep anti-ANXA1 polyclonal antibody (dilution, 1:1000) and for 1 h with antisheep IgG-15-nm gold complex, and then lightly counterstained with uranyl acetate and lead citrate. All antibodies were diluted in 0.1 M phosphate buffer containing 0.1% egg albumin. For control sections, the primary antibody was replaced with anti-ANXA1 serum that had been preincubated with an excess of human recombinant ANXA1 (a gift from Dr. J. Browning, Biogen Research Corp., Cambridge, MA). Sections were examined with a JEOL 1010 transmission electron microscope (JEOL USA, Inc., Peabody, MA).

Isolation of primary adrenal cells

Isolated adrenal cells were prepared by a modification of Pedersen and Brownie (24). Briefly, adrenal glands were diced and digested in collagenase (0.4 mg/ml, wt/vol) (Worthington, Lakewood, NJ) and pancreatin (0.6 mg/ml, wt/vol) (Sigma) in MEM for 40 min at 37 C with agitation; the dispersion was aided by gentle trituration. The resulting cell suspension was added to 100% FCS to produce a final concentration of 5% FCS (Life Technologies, Inc., Paisley, UK). The cells were centrifuged for 10 min at 2000 × *g*, the supernatant removed, and the cells resuspended in 5% FCS in MEM. The cells were examined at the light microscope level to determine the effectiveness of the dispersion and counted using a hemocytometer. Cell viability was assessed using the trypan blue (Sigma) exclusion test and always found to be greater than 97% viability. In some experiments, the cells were retained for counting of cortical lipid-droplet-containing cells and granulated medullary cells by electron microscopy. Briefly, cells were postfixed in 1% osmium tetroxide in 0.1 M phosphate buffer, stained in 2% uranyl acetate in distilled water, dehydrated through a series of increasing ethanol concentrations, and embedded in Spurr's resin. Ultrathin sections were prepared as above, and the cell numbers counted blind in six sections (500 cells were counted per section) taken from different depths of the embedded cell pellet.

Static incubation of primary adrenal cells

Wild-type and ANXA1-null primary adrenal cells were plated out at a density of 50,000 cells/ml per well in 24-well cell culture plates (Costar, High Wycombe, UK) in MEM supplemented with 5% FCS, 0.5% penicillin/streptomycin, and 0.5% glutamine and incubated for 1 h at 37 C, 95% O_2/5% CO_2. For the stimulation period (1 h), the medium was changed to medium containing either $ACTH_{1–24}$ (0.01–5 nM) (Bachem Ltd., Saffron Walden, UK) or angiotensin II (1–100 nM) (Sigma). Controls were incubated in an equal volume of medium alone. After the stimulation period, the culture plates were centrifuged (600 × *g* for10 min) and the supernatant removed for measurement of corticosterone or aldosterone. The viability of the cells was tested by trypan blue exclusion and found to be more than 97%. In additional experiments, wild-type adrenal cells were incubated in basal and ACTH-stimulated (100 pM) conditions in the presence of graded concentrations of the N-terminal peptide $ANXA1_{Ac2–26}$ (0.003–30 μM) or in negative controls, boiled peptide Ac2–26 (0.003–30 μM). Peptide $ANXA1_{Ac2–26}$ (acetyl-AMVSEFLKQAWIENEEQEYVVQTVK; molecular weight, 3050) was prepared by the Advance Biotechnology Centre, Charing Cross Campus, Imperial College (London, UK), by using solid-phase stepwise synthesis (kindly provided by Prof. Mauro Perretti, William Harvey Research Institute, London, UK). Purity was more than 90% as assessed by HPLC and capillary electrophoresis.

RIA for corticosterone and aldosterone

Plasma corticosterone concentrations were determined in duplicate by use of a commercially available RIA (IDS Ltd., Tyne and Wear, UK), according to the manufacturer's instructions. The inter- and intraassay coefficients of variation were 3.1 and 5.8%, respectively. Plasma aldosterone concentrations were also determined in duplicate by use of a commercially available RIA (Diagnostic Systems Laboratory Inc., Webster, TX), according to the manufacturer's instructions. The inter- and intraassay coefficients of variation were 4 and 9%, respectively, and the minimum detection level of the assay was 8 pg/ml.

Morphometric analysis of wild-type and ANXA1-null adrenals

Adrenal glands were fixed for 24 h in Bouin's solution, embedded in paraffin, and serially sectioned at 5 μm. Sections were stained with hematoxylin and eosin by standard protocols and photographed using an Olympus BH microscope fitted with an Axiovision digital camera (Zeiss, Hemel Hempstead, UK). The area of the cortex was analyzed from digital images using Image J software (National Institutes of Health, Bethesda, MD; http//rsb.info.nih.gov/ij). The measurement was performed on five adjacent sections from the middle portion of each individual adrenal gland to ensure a reliable comparison. The cortical area was normalized for the medullary area and expressed as the cortical/medullary area ratio (n = 4 animals). Cell nuclei within the zona fasciculata of four sections from wild-type and ANXA1 mice (n = 4 animals) were counted under standardized conditions. Cell counts were expressed as cell number per high-power field (HPF).

Data analysis

Semiquantitative measures of ANXA1 and β-actin expression were made by comparisons of Western blot band OD (arbitary units) to give a relative numerical guide to the ratios of the band intensities and their variance. Responses to dexamethasone were calculated as a ratio of the drug-free control and expressed as the mean ± SEM (n = 3 gels). Statistical comparisons between the normally distributed data were made by ANOVA. For data from the *in vitro* isolated adrenal cell release studies, all values represent the mean ± SEM (n = 6 per group). Preliminary analysis confirmed that the data were normally distributed. Subsequent analysis was undertaken by one-way ANOVA with *post hoc* analysis performed using Duncan's multiple range test. In all cases,

differences were considered significant if $P < 0.05$. Each of the studies was repeated several times, and in all instances the data profile was similar.

Results

RT-PCR and Western blot analysis of ANXA1 in the adrenal gland

Initial studies demonstrated that ANXA1 mRNA and protein were readily detectable in the adrenal gland by RT-PCR and Western blot analysis, respectively. RT-PCR using primers against ANXA1 mRNA detected a band of the expected size (1062 bp) in adrenal gland tissue (Fig. 1A). Real-time PCR revealed that the adrenal gland expressed ANXA1 mRNA in relatively large amounts compared with the positive control tissues tested, in decreasing order of expression as follows: thymus more than adrenal more than spleen more than pituitary more than brain (Fig. 1C). Figure 1A illustrates the relative amount of ANXA1 mRNA expression in the adrenal (lane 3) compared with anterior pituitary (lane 1), and Fig. 1B illustrates the GAPDH-negative controls for anterior pituitary (lane 2) and adrenal (lane 3), showing no difference in the amount of GAPDH mRNA detected. Western blot analysis detected a major band corresponding to the native biologically active 37K species of the protein (Fig. 2). In some experiments, a second band of approximately 32K (which in other tissues is reported to represent a metabolite (3) was also detected (Fig. 2C). Figure 2A demonstrates that the ANXA1 immunoreactivity was retained in tissue collected from saline-perfused tissue (lanes 3 and 4, perfused, *vs.* lanes 1 and 2, nonperfused), excluding the possibility that the ANXA1-immunoreactive bands were derived from circulating leukocytes. Figure 2, B–D, illustrates the effect of dexamethasone (0.1 μM, 3 h) on the cellular location of ANXA1 in adrenal gland segments. Typical Western blots are shown in Fig. 2, B and C, and densitometry data in Fig. 2D. Dexamethasone treatment significantly ($P < 0.01$) increased the amount of ANXA1 detected in both the EDTA wash and intracellular lysate of adrenal segments (Fig. 2, B and C, control lanes 1 and 2, *vs.* dexamethasone-treated lanes 3 and 4; densitometry shown in Fig. 2D). No change was observed in the amount of β-actin quantified in intracellular extracts (Fig. 2D), and no β-actin-immunoreactive bands were detected in cell surface EDTA washes (data not shown).

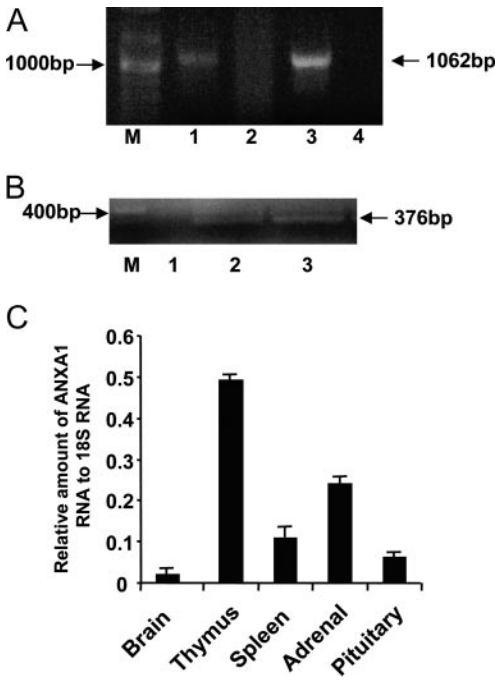

FIG. 1. A, RT-PCR gel showing detection of ANXA1 mRNA in the adrenal gland: lane 1, anterior pituitary (positive control); lane 2, anterior pituitary negative control (no RT enzymes in RT-PCR); lane 3, adrenal; lane 4, adrenal negative control. M, Size markers. B, GAPDH internal control: lane 1, adrenal negative control; lane 2, adrenal; lane 3, anterior pituitary. C, Histogram showing the relative expression of ANXA1 mRNA to 18S mRNA measured by real-time PCR in adrenal gland, thymus, spleen, pituitary, and brain.

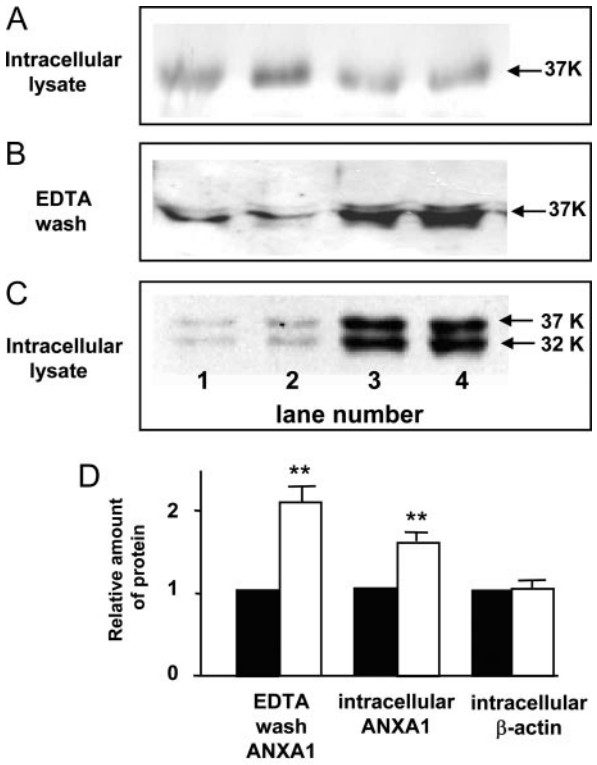

FIG. 2. A, Western blot showing detection of ANXA1 in nonperfused (lanes 1 and 2) and saline-perfused (lanes 3 and 4) adrenal gland tissue. B and C, Western blots showing the effect of dexamethasone (Dex; 0.1 μM, 3 h) on EDTA wash of cell surface ANXA1 (B) and intracellular ANXA1 in adrenal gland tissue (C). Controls are in lanes 1 and 2, dexamethasone-treated in lanes 3 and 4. D, Histogram showing integrated densitometry data for the effect of dexamethasone on ANXA1 and β-actin. Values are expressed as mean ± SEM; n = 3 experiments. *Black bars* represent controls; *white bars* represent dexamethasone-treated. **, $P < 0.01$ (ANOVA).

Determination of ANXA1 localization in the adrenal gland by light and electron microscopy

Strong immunofluorescent staining for β-Gal in ANXA1-null adrenal sections (Fig. 3A) was restricted to the capsule and outer cortical cells. The adrenal medulla and majority of the cortical zone was unstained. No β-Gal immunofluorescence was detected in wild-type adrenal sections (Fig. 3B). Intense ANXA1 immunofluorescence in wild-type adrenal

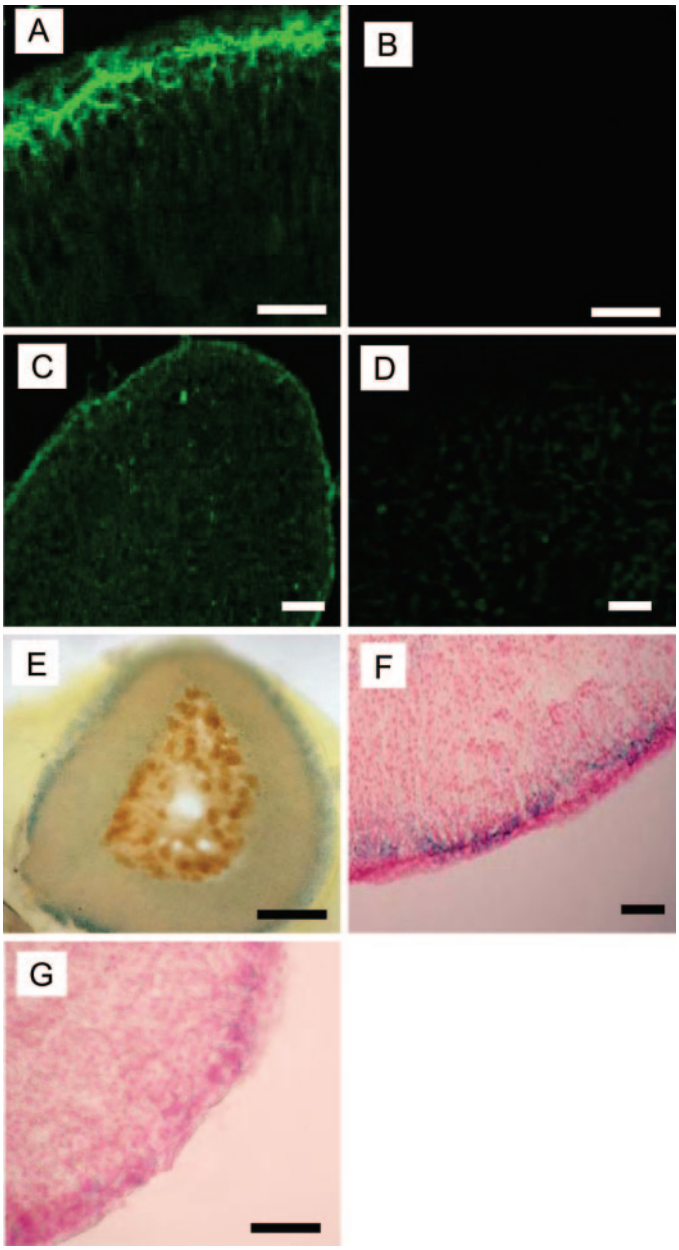

FIG. 3. Light microscopy showing ANXA1 localization to be restricted to the perimeter cell layers of the adrenal gland. A and B, Immunofluorescence showing β-Gal transgene immunoreactivity in a section of ANXA1-null mouse adrenal (A) and absence of β-Gal immunoreactivity in wild-type mouse adrenal (B); C and D, immunofluorescence showing ANXA1 immunoreactivity in a section of wild-type adrenal (C) and negative control showing absence of intense perimeter immunolabeling in an ANXA1 absorbed-primary-antibody-incubated section of wild-type adrenal (D); E–G, X-gal histochemistry in ANXA1-null adrenal confirming localization to the perimeter of the gland (E and F) and absence of X-gal reaction product in a section of wild-type adrenal gland (G). *Scale bars*, 100 μm (A–D, F, and G) and 1 mm (E).

sections (Fig. 3C) was also detected in the capsule and outer cortical cell layers with weaker immunoreactivity scattered throughout the gland. In control sections incubated with ANXA1 absorbed antibody (Fig. 3D, negative controls) the intense capsular/outer cortical labeling was lost, but weak scattered immunolabeling within the deeper cortical layers remained, indicating that this was likely to be nonspecific labeling by the antisera. X-Gal histochemistry confirmed the immunofluorescence data (Fig. 3, E and F). In ANXA1-null adrenal sections, blue X-Gal reaction product was concentrated in the outer cell layers (Fig. 3E, low power, and F, high power); no reaction product was visualized in wild-type sections (Fig. 3G). In ANXA1-null adrenal sections, no labeling was evident in the adrenal medulla (Fig. 3E). Electron microscopy and immunogold localization of subcellular ANXA1 in wild-type tissue demonstrated diffuse ANXA1 immunoreactivity over the cytoplasm and nuclei of adrenal capsule cells (Fig. 4A) and outer zona glomerulosa steroid-producing cells (Fig. 4B). No significant accumulation of immunogold at the plasma membrane was visualized. Preincubation of the anti-ANXA1 serum with an excess of human recombinant ANXA1 abolished the immunostaining (Fig. 4, C and D), demonstrating that the labeling was specific.

Effect of ACTH on corticosterone release from wild-type and ANXA1-null isolated adrenal cells in vitro

Figure 5A demonstrates that no significant difference in basal corticosterone (Fig. 5A) output from wild-type and ANXA1-null adrenal cell preparations was measured *in vitro*. In both wild-type and ANXA1-null adrenal cells, ACTH (0.1–50 nM) produced the expected stimulation ($P < 0.05$) of corticosterone release. However, corticosterone responses to ACTH were significantly greater ($P < 0.05$) in ANXA1-null adrenal cell preparations (Fig. 5A; EC_{50} for ACTH = 480 ± 62 pM in wild-type cells *vs.* 57 ± 14 pM in ANXA1-null cells). No significant difference in aldosterone release between wild-type and ANXA1-null cells were measured in basal, ACTH-stimulated (Fig. 5B), or angiotensin II-stimulated (1–100 nM; Fig. 5C) conditions. Cell viability, as assessed by trypan blue exclusion test, was well maintained (>97%) at the end of the static incubations. Adrenal MC2R, GR, and GAPDH mRNA was detected as appropriately sized bands by RT-PCR, whereas the negative control reactions yielded no band (Fig. 6, A–C). No difference in the amount of adrenal MCR2 (Fig. 6A) and GR (Fig. 6B) mRNA or anterior pituitary GR mRNA in wild-type and ANXA1-null mice was detected. Figure 6C shows the GAPDH mRNA internal control.

Figure 7 demonstrates the effects of the ANXA1 N-terminal peptide $ANXA1_{Ac2–26}$ (0.003–30 μM) on basal and ACTH-stimulated corticosterone (Fig. 7A) and aldosterone release (Fig. 7B) from isolated wild-type adrenal cells *in vitro*. $ANXA1_{Ac2–26}$ did not influence basal release of corticosterone (Fig. 7A) or basal or ACTH-stimulated aldosterone release (Fig. 7B). However, $ANXA1_{Ac2–26}$ produced a concentration-dependent inhibition of the ACTH-stimulated release of corticosterone (Fig. 7A), whereas incubation of cells with boiled $ANXA1_{Ac2–26}$ had no effect on basal or ACTH-stimulated release (Fig. 7A). $ANXA1_{Ac2–26}$ produced a maximal 30–40% inhibition of ACTH-evoked corticosterone release and was effective in the micromolar range.

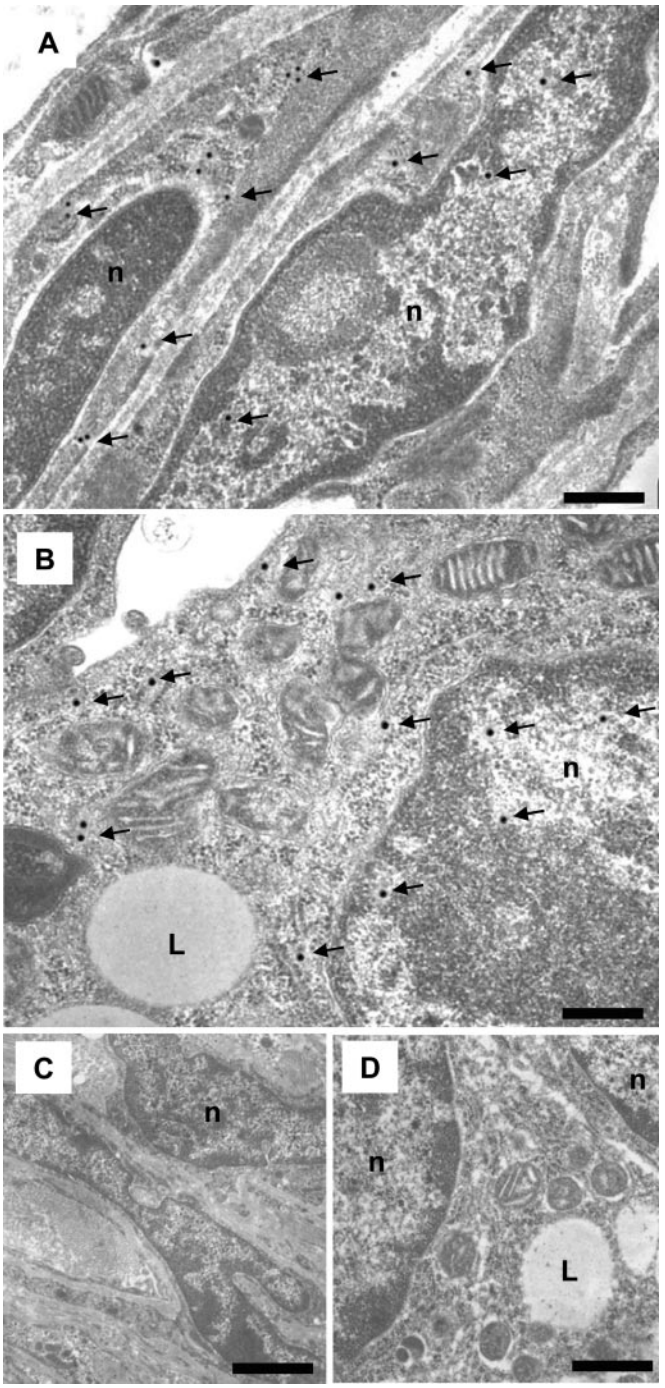

FIG. 4. A and B, Electron micrograph from a freeze-substituted wild-type mouse adrenal gland section showing immunogold detection of ANXA1 in adrenal capsule cells (A) and zona glomerulosa steroidogenic cells (B). In both cell types, gold particles (15 nm; *arrows*) were scattered over the cytoplasm and nucleus. C and D, Electron micrographs of adrenal capsule (C) and of zona glomerulosa cells (D) illustrate the absence of immunogold labeling in negative controls incubated with anti-ANXA1 serum preincubated with an excess of human recombinant ANXA1. *Scale bars*, 1 μm (A and B) and 2 μm (C and D). L, Lipid droplet; n, nucleus.

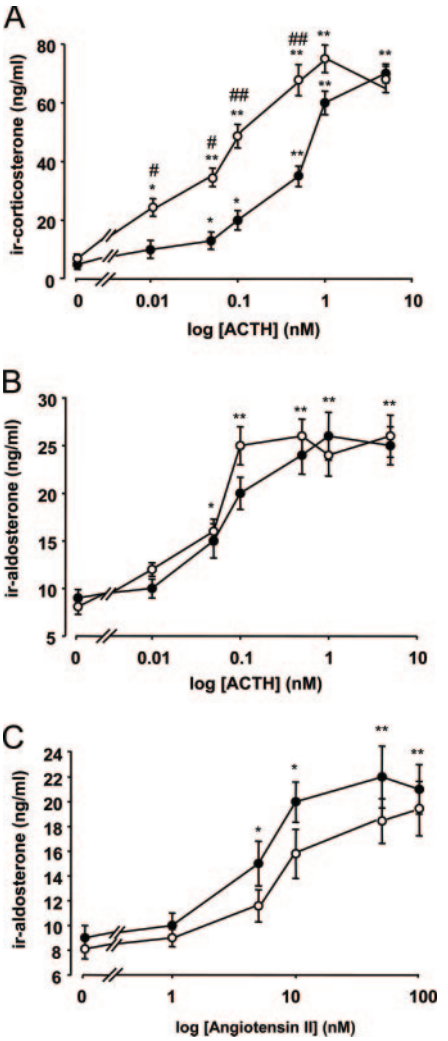

FIG. 5. A and B, Effects of graded concentrations of ACTH (0.01–5 nM) on the release of immunoreactive (ir)-corticosterone (A) and aldosterone (B) from wild-type and ANXA1-null isolated adrenal cells *in vitro*; C, effects of graded concentrations of angiotensin II (1–100 nM) on the release of aldosterone from wild-type and ANXA1-null isolated adrenal cells. ●, Wild-type cells; ○, ANXA1-null cells. Values represent the mean ± SEM; n = 6. **, $P < 0.01$, *, $P < 0.05$ *vs.* control; #, $P < 0.05$; ##, $P < 0.01$ *vs.* corresponding treatment in wild-type (ANOVA and Duncan's multiple range test). Typical data are shown from three replicate experiments.

Wild-type and ANXA1-null adrenal gland histology

A decrease in adrenal cortical thickness was evident in ANXA1-null mice compared with wild-type mice on histological examination (Fig. 8, A and B). Quantitation of the cortical/medullary area ratios demonstrated a significant decrease ($P < 0.01$) in ANXA1-null mice (Fig. 8C). Cell counts per HPF in the zona fasciculata were not significantly different in ANXA1-null mice compared with wild-type mice, indicating no change in cell size (Fig. 8D). The proportion of lipid-droplet-containing cells (cortical) and granulated medullary cells were counted by electron microscopy in wild-type and ANXA1-null isolated adrenal cell preparations. ANXA1-null adrenal cell preparations contained a significantly ($P < 0.05$) lower proportion of cortical cells compared with wild-type (wild-type, 71 ± 5% cortical cells; ANXA1-null, 54 ± 7% cortical cells; n = 4 cell preparations).

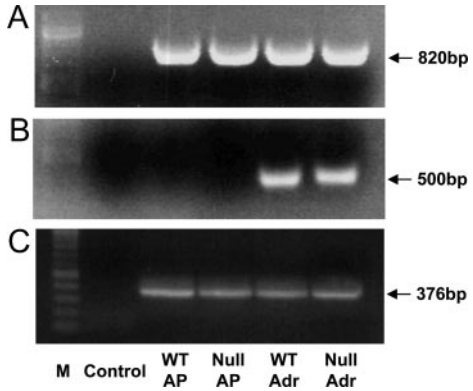

FIG. 6. RT-PCR gels demonstrating GR (A), MC2R (B), and GAPDH (C) (internal control) mRNA in wild-type and ANXA1-null anterior pituitary (AP) and adrenal glands (Adr). Bands of the predicted size were observed, and no differences in wild-type and ANXA1-null tissue expression were observed. M, Size markers; control, no RT enzymes in PCR.

Discussion

The results presented demonstrate the expression and localization of ANXA1 in the murine adrenal gland and used tissue from ANXA1-null and wild-type mice to explore the function of ANXA1 in the adrenal gland. ANXA1 is expressed in abundance in peripheral blood leukocytes, particularly monocytes and neutrophils (1), and for this reason, saline perfusion was performed before tissue analysis of ANXA1 expression. After perfusion, strong ANXA1 mRNA and protein expression remained in the adrenal as detected by RT-PCR and Western blot analysis, respectively. As in several other tissues (3, 8, 9), the expression and subcellular distribution of ANXA1 in adrenal cells was regulated by glucocorticoid treatment. Treatment with the synthetic glucocorticoid dexamethasone for 3 h stimulated a significant increase in both surface and intracellular ANXA1 protein as detected by Western blot analysis. Immunohistochemical studies and visualization of β-Gal reporter activity localized ANXA1 to the peripheral cortical cell layers of the adrenal gland. These data were confirmed by immunogold electron microscopy, which localized ANXA1 immunoreactivity specifically to capsular cells and outer cortical steroidogenic cells identified by lipid droplets. Within these cells, the majority of intracellular ANXA1 appeared to be free within the cytosol, consistent with our findings in other tissues (8). The electron microscopic analysis confirmed that ANXA1 is a very labile protein because freeze-substitution methods were required to detect ANXA1 immunoreactivity in the adrenal. This repeats our finding for the preservation of ANXA1 immunoreactivity in pituitary folliculostellate cells (8), transfected AtT20 cells (12), and lung adenocarcinoma A549 cells (25).

Besides the classical corticotrophic hormones, ACTH and

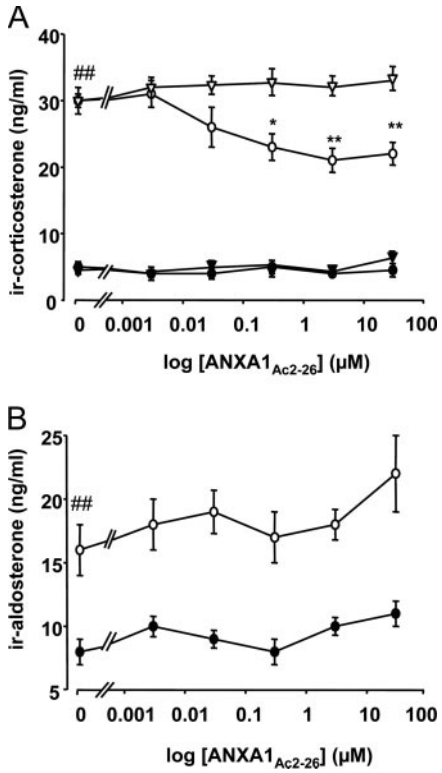

FIG. 7. A and B, Effect of $ANXA1_{Ac2-26}$ (0.003–30 μM) on basal and ACTH-stimulated (100 pM) corticosterone (A) and aldosterone (B) release: ○, ACTH-stimulated groups in presence or absence of $ANXA1_{Ac2-26}$; ●, basal groups in presence or absence of $ANXA1_{Ac2-26}$. In A, boiled $ANXA1_{Ac2-26}$ controls are shown: △, ACTH-stimulated groups in presence or absence of boiled $ANXA1_{Ac2-26}$; ▲, basal groups in presence or absence of boiled $ANXA1_{Ac2-26}$. Each value represents the mean ± SEM; n = 6. *, $P < 0.05$; **, $P < 0.01$ *vs.* ACTH-alone control; ##, $P < 0.01$ *vs.* corresponding ACTH-free control (ANOVA plus Duncan's test). ir, Immunoreactive.

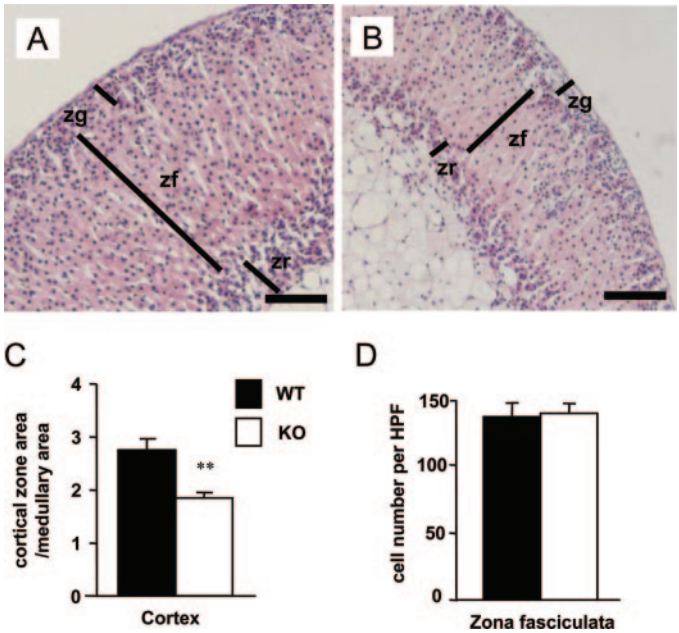

FIG. 8. A and B, Micrographs of hematoxylin- and eosin-stained adrenal sections from a wild-type animal (A) and ANXA1-null mouse (B). Note the decreased adrenocortical width in the ANXA1-null mouse compared with wild-type. C, Histogram showing cortical/medullary area ratios in wild-type and ANXA1-null adrenal glands. D, Histogram showing cell counts per HPF within the zona fasciculata of adrenals from wild-type and ANXA1-null mutant mice. Each value represents the mean ± SEM; n = 4 animals. *Scale bars*, 200 μm. **, $P < 0.01$ (ANOVA).KO, Knockout; zf, zona fasciculata; zg, zona glomerulosa; zr, zona reticularis.

angiotensin II, various regulatory peptides produced by the adrenal gland are thought to participate in the control of corticosteroid secretion (26). In the neuroendocrine and host defense systems, the process of ANXA1 externalization enables ANXA1 to serve as a paracrine mediator of glucocorticoid action, and it is possible that a similar role exists in the adrenal. The experimental single adrenal cell preparation we used, in which adrenal cells were dispersed by mild enzymatic treatment and trituration, has been used widely in the rat to examine the mechanisms controlling secretion of adrenal steroids. Our preliminary studies using mouse adrenal glands revealed that the dispersed murine adrenal cells were well preserved morphologically and responded to ACTH with concentration-dependent increases in immunoreactive corticosterone. No significant difference in basal secretion of corticosterone was measured in wild-type and ANXA1-null mice. This result is consistent with the normal circulating plasma ACTH and corticosterone concentrations measured in ANXA1-null mice (27). However, the response to ACTH was significantly greater in ANXA1-null cells compared with wild-type, suggesting that ANXA1 exerts inhibition of stimulated corticosterone output. Consistent with this, $ANXA1_{Ac2–26}$ concentration-dependently inhibited corticosterone release. Although a maximal inhibition of 30–40% of ACTH-stimulated corticosterone release was achieved in response to $ANXA1_{Ac2–26}$, it is possible that the full-length ANXA1 may be of greater efficacy, as previously observed (28). Basal and angiotensin II-stimulated aldosterone secretion was not significantly different from wild-type and ANXA1-null cell preparations and was not influenced by $ANXA1_{Ac2–26}$. The same numbers of isolated cells were incubated in wild-type and ANXA1-null adrenal cell preparations, but the proportion of cortical cells was reduced in the ANXA1-null preparation compared with wild-type. However, despite this, we measured an increase in corticosterone release. Therefore, it would seem the responsiveness of the cortical cells to the secretagogues has increased. No difference in MC2R and GR mRNA expression between wild-type and ANXA1-null cells was observed, but it is possible that ANXA1 may influence second messenger signaling downstream of the receptors. The lack of a difference in basal corticosterone levels between wild-type and ANXA1-null mice may reflect the possibility that only a small proportion of cortical cells contribute to basal release. ANXA1 is well established as a paracrine mediator of glucocorticoid intermediate feedback on corticotrope ACTH release in the anterior pituitary (2–5), and a local CRH/ACTH system in the adrenal has been characterized with adrenal medullary cells being the source of ACTH (26), which might similarly be regulated by ANXA1. Another potential candidate for interaction is IL-6, which also augments corticosterone production and in other tissues (*e.g.* hypothalamus) produces effects that are blocked by ANXA1 (7, 26). Additional experiments are required to explore the possibility that ANXA1 may exert similar inhibitory control of these systems in the adrenal.

A decrease in adrenal cortical depth was observed in ANXA1-null tissue, but no significant difference in the cell number per HPF in the zona fasciculata was measured. These measurements suggest that ANXA1 has a role in the regulation of adrenal gland cell proliferation rather than growth. The concentration of ANXA1 immunoreactivity to capsular and outer cortical cells would be consistent with this possibility because cortical cells are derived from progenitor cells (in the mouse, shown to be glomerulosa cells adjacent to the capsule) (32), which migrate and populate the inner cortical zones (29–32). However, the results were surprising because there is strong evidence for ANXA1 inhibition of cell proliferation in a lung adenocarcinoma cell line (A549), mesangial cells, and lymphocytes (33–36). Furthermore, we have recently revealed a 4-fold increase in the number of pituitary corticotropes in ANXA1-null male mice (27), although paradoxically, male rats in which pituitary ANXA1 expression and membrane translocation is suppressed by perinatal dexamethasone treatment show a 50% reduction in corticotrope number (37). Collectively, these reports would predict adrenal cortical hypertrophy in the ANXA1-null mouse and not atrophy as we have shown. It is unlikely that the decrease in adrenal size is secondary to alterations in the hypothalamo-pituitary axis in the ANXA1-null mouse because circulating basal ACTH and corticosterone concentrations are normal (27) in these animals. Although thymic weights are reduced in tissue mass in relation to body weight in ANXA1-null mice, breeding statistics, body weight, and growth rates are normal (38). It is possible that alterations in the amount of other annexin family members may contribute to the phenotype because ANXA1 gene deletion has been shown to produce tissue-dependent changes in the expression of other annexins (38). Little is known with regard to the expression of annexin subtypes in the adrenal gland. However, ANXA7, a Ca^{2+}-activated GTPase, is associated with exocytosis from adrenal medulla chromaffin cells. Deletion of ANXA7 is a lethal mutation, and although the heterozygote animals are viable, the adrenal glands display hypertrophy and chromaffin cell hyperplasia (39) but no cortical abnormalities.

In conclusion, the present report adds ANXA1 to the list of putative agents produced and externalized by outer cortical adrenal cells that regulate corticosterone secretion. In contrast, aldosterone secretion was not affected in ANXA1-null adrenal cells or modulated by $ANXA1_{Ac2–26}$. Additional studies are underway to determine whether ANXA1 is a mediator of glucocorticoid action in the adrenal gland as in other tissues.

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