The identity of the putative G-protein coupled receptor (GPCR) that mediates the non-genomic effects of androgens is unknown. We present in vitro and in vivo evidence that the orphan GPRC6A receptor, a widely expressed calcium and amino acid sensing GPCR, transduces the non-genomic effects of testosterone and other steroids. Overexpression of GPRC6A imparts the ability of extracellular testosterone to illicit a rapid, non-genomic signaling response in HEK-293 cells lacking the androgen receptor. Conversely, testosterone-stimulated rapid signaling and phosphorylation of ERK is attenuated in bone marrow stromal cells derived from GPRC6A−/− mice and in 22Rv1 prostate cancer cells after siRNA-mediated knockdown of GPRC6A. Compared with wild-type controls, GPRC6A−/− null mice exhibit significantly less ERK activation and Egr-1 expression in both bone marrow and testis in response to pharmacological doses of testosterone in vivo. In addition, testosterone administration results in suppression of luteinizing hormone in wild-type male mice, but paradoxically stimulates serum luteinizing hormone levels in GPRC6A−/− null mice. These results suggest that GPRC6A is functionally important in regulating non-genomic effects of androgens in multiple tissues.

Androgens are important regulators of reproductive physiology and anabolic biological activities in multiple tissues (1). In addition, androgens also play a role in the pathogenesis of prostate cancer, including disease severity, progression, and metastasis (2, 3). The classical genomic actions of androgens are mediated through the androgen receptor (AR), a member of the nuclear receptor superfamily of transcription factors (4–6). Androgen binding to AR and translocation of the steroid-receptor complex to the nucleus regulates steroid response elements in promoters to modulate gene expression over a period of hours (7). Androgens and selective androgen receptor modulators are being explored as therapy for age-related conditions, such as osteopenia and sarcopenia (8). In addition, androgens illicit rapid (occurring in minutes) non-genomic effects that may also have important biological effects in many tissues (5, 9–11). The molecular mechanisms underlying these non-genomic actions are poorly understood. Several mechanisms have been proposed, including translocation of the steroid receptors to the cell surface membrane (12–14), nonspecific effects of steroids on the fluidity of lipids in the plasma membrane, direct allosteric modification of ligand-gated ion channels (5), and activation of G-protein coupled receptors (GPCRs) (9, 15, 16), including a putative pertussis toxin-sensitive GPCR (17–21). Identification of the putative GPCR that transduces the non-genomic effects of androgens may add significantly to our understanding of androgen biology that is relevant to normal human development and disease as well as the health risks of excessive pharmacological doses of anabolic steroids used in body building.

GPRC6A is a pertussis toxin-sensitive member of the C family of GPCRs that senses amino acids, extracellular calcium, and osteocalcin (22–24). GPRC6A may function as an anabolic receptor coordinating the responses of multiple tissues to changes in nutrients and other factors (25). In this regard, ablation of this orphan G-protein coupled receptor leads to testicular feminization in male mice, suggesting that GPRC6A may also modulate sex steroid end organ responses (25).

In the present study, we examined the role of GPRC6A in mediating the non-genomic effects of androgens on cell function in vitro and in vivo. We found that GPRC6A mediates the rapid signaling response to testosterone and other steroids in various cell culture models. Indeed, cells isolated from GPRC6A null mice failed to exhibit rapid signaling responses to androgens, and prostate cells expressing endogenous GPRC6A attenuated their non-genomic responsiveness to androgens following siRNA knockdown of GPRC6A. More importantly, exogenously administered testosterone exhibited an impaired rapid response in vivo of GPRC6A null mice and failed to fully restore seminal vesicle size, as a measure of androgen tissue responsiveness, in orchectomized GPRC6A null mice. Testosterone, which suppresses luteinizing hormone (LH) levels in control mice, paradoxically stimulated LH levels in GPRC6A null mice. Finally, loss of GPRC6A retarded the progress and improved survival of a mouse model of prostate
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cancer. Together, our findings indicate that GPRC6A is a candidate for a biologically relevant androgen sensing GPCR.

EXPERIMENTAL PROCEDURES

Reagents—Testosterone, dihydrotestosterone, dehydroandrosterone, testosterone-3-(O-carboxymethyl)oxime: BSA (testosterone/BSA), testosterone-3-(O-carboxymethyl)oxime/BSA/fluorescein isothiocyanate conjugate (testosterone/BSA/FITC) (10 mol of testosterone/mol of BSA), BSA/FITC, β-estradiol, progesterone, bicalutamide, and flutamide were obtained from Sigma. Methytrienolone (R1881) was purchased from PerkinElmer Life Sciences. In the case of testosterone/BSA, before each experiment, stock solutions of BSA conjugates were mixed with dextran (0.05 mg/ml) and charcoal (50 mg/ml) for 30 min, centrifuged at 3000 × g for 10 min, and passed through a 0.22-mm pore size filter to remove any potential contamination with free testosterone.

Mouse—Mice were maintained and used in accordance with recommendations as described by the National Research Council (Guide for the Care and Use of Laboratory Animals DHHS Publication NIH 86–23, Institute on Laboratory Animal Resources) and following guidelines established by the University of Tennessee Health Science Center Institutional Animal Care and Use Committee. The GPRC6A-deficient mouse model was created by replacing exon 2 of the GPRC6A gene with the hygromycin resistance gene (25). For genotyping GPRC6A+/− mice, the PCR primers were Athx-1, GAATAACTAGCAAGGGGCGCTGAAGAGG and Athx-2, CAGAGTGGCAGCCATTGGCTGCTGTGACTTCG (wild type pair); Athx-α, CAGAGGATCGTGGGTATCATGCACAG and Athx-β, CTACATGGCCGTATTTATCATGTGGCTTGGC (Hygro knock-out pair).

RT-PCR—RT-PCR was performed using a two-step RNA PCR protocol (PerkinElmer Life Sciences). In separate reactions, 2.0 μg of DNase-treated total RNA was reverse transcribed into cDNA with the reverse primers and Moloney murine leukemia virus reverse transcriptase (Invitrogen). Reactions were carried out at 42 °C for 60 min followed by 94 °C for 5 min and 5 °C for 5 min. The products of the first strand cDNA synthesis were directly amplified by PCR using AmpliTaq DNA polymerase (PerkinElmer Life Sciences). The PCR primer sets used to amplify various gene transcripts with in-tron-spanning are as follows: hGPRC6A.F203, CAGGAGTGTTGGCATTCTGTTTG and hGPRC6A.R630, ATCAGGTGTGTTGCTTT; mGPRC6A.Rev1427, TGGGCATCAAAGTGAGCCATTGCTTT; mGPRC6A.For425, ATTCCAGCTACGTTGTTGGCTTTGA and hGPRC6A.R630, ATCAGGTGTGTTGCTTT; mGPRC6.Rev1779, GGACTTGTGCATGCGGTAC-TGCCAAGG and mGPRC6.For438, AACACTTTGTGGCCTGAACC and hGPRC6A.F143, GACCCCTTCATTGACCTCAACTACA and Egr1.Rev897, ACTGAGTGGCGAAGGCTTTA; TCA; Egr1.For438, AACACTTTGTGGCCTGAACC and ACAC and hAR.Rev1779, GGACTTGTGCATGCGGTAC-TGCCAAGG and mGPRC6.Rev1427, TGGGCATCAAAGTGAGCCATTGCTTT; mGPRC6.For425, ATTCCAGCTACGTTGTTGGCTTTGA and hGPRC6A.R630, ATCAGGTGTGTTGCTTT.

Cell Culture and Luciferase Activity Assay—HEK-293 cells were co-transfected with pcDNA3.mGPRC6A and SRE-luciferase reporter gene plasmids. Quiescence of transfected cells was achieved in subconfluent cultures by removing the medium and washing with Hanks’ balanced salt solution (Invitrogen) to remove residual serum followed by incubation for an additional 24 h in serum-free quiescent DMEM/F-12 medium (Invitrogen, catalog number 11330; 1 mM calcium). For studies that used calcium and amino acids to activate GPRC6A, we used DMEM (Invitrogen, catalog number 21068; containing no calcium and 0.4 mM arginine) for both the quiescent and stimulation phases of the studies. Luciferase activity was assessed after 6 h of stimulation in cell extracts of a luciferase assay system (Promega) and a BG-luminometer (Gem Biomedical, Inc.). The values depicted represent the mean ± S.E. of a minimum of three separate experiments.

The prostate cancer cell line, 22Rv1, was obtained from the American Type Culture Collection (Manassas, VA) and maintained in RPMI 1640 supplemented with 10% fetal bovine serum. Cells (105 cells/well) were cultured in triplicate in a 96-well flat-bottomed microculture dish in the presence and absence of various concentrations of GPRC6A ligands, including calcium, amino acids, testosterone, and osteocalcin for 72 h. Cell proliferation was determined by counting cells with a hemocytometer (26).

Western Blot—HEK-293 cells were co-transfected with pcDNA3.mGPRC6A or pcDNA3 or pcDNA3.rCaSR plasmid as previously described (22). Quiescence was achieved in subconfluent cultures by removing the medium and washing with Hanks’ balanced salt solution (Invitrogen) to remove residual serum, followed by incubation for an additional 24 h in serum-free medium (DMEM/F-12 medium containing 0.1% BSA and about 1 mM calcium; Invitrogen, catalog number 11330). After agonist treatment at the specified concentrations and duration, cells were washed twice with ice-cold PBS and scraped into 250 μl of lysis buffer (25 mM HEPES, pH 7.2, 5 mM MgCl2, 5 mM EDTA, 1% Triton X-100, 0.02 tablet/ml of protease inhibitor mixture). Equal amounts of lysates were subjected to 10% SDS-PAGE, and phospho-ERK1/2 levels were determined by immunoblotting using anti-phospho-ERK1/2 mitogen-activated protein kinase antibody (Cell Signaling Technology). To confirm that variations in the amount of ERK did not contribute to stimulated ERK activity, we used an anti-ERK1/2 mitogen-activated protein kinase antibody (Cell Signaling Technology) to measure ERK levels. Antibodies for c-Src, phospho-Src, and phospho-Raf-1 were purchased from Cell Signaling Technology. Representative blots are shown of the results that were verified at least in three independent experiments.

Computational Modeling—Sequences of the human metabotropic glutamate receptor, calcium-sensing receptor, and GPRC6A were obtained from GenBank™ and aligned against the crystalized sequences of the β2 adrenergic receptor (β2, Protein Data Bank entry 2RH1) (27, 28), rhodopsin (PDB entry 1F88) (29), β1 adrenergic receptor (β1, PDB entry 2VT4) (30), and adenosine A2a receptor (PDB entry 3EML) (31) using MOE (Chemical Computing Group, Montreal, Canada, version 2009.10) software. The three class C receptors and the four class A receptors were separately aligned before the two classes were aligned using group-to-group alignment.

Homology models of the transmembrane bundle of GPRC6A were developed from the group-to-group alignment.
using both β1 and rhodopsin as templates. In each case, 10 different backbone models were generated and three sets of side chain conformers were sampled for each backbone model. The best-scored model from each template was used as a docking target for testosterone using the extracellular loops and upper third of the transmembrane bundle as the docking site. Representative positions of testosterone are presented to demonstrate that sufficient space surrounded by hydrophobic side chains is available in both models.

Labeling with Testosterone/BSA/FITC—Cell surface binding of testosterone was evaluated by modifications of previously described methods (18). Briefly, HEK-293 cells stably expressing GPRC6A or untransfected HEK-293 cells were grown on glass coverslips for 48 h, washed with PBS, and then incubated with 15 μM testosterone/BSA/FITC (Sigma) at room temperature for 5 to 10 s, followed by two washings with PBS and cell fixation with 2% paraformaldehyde for 30 min. The cellular distribution of testosterone/BSA/FITC was then determined by fluorescent microscopy. BSA/FITC and BSA were used in the corresponding control experiments.

Binding Assay—Binding assays were performed as previously described (32). Briefly, membranes from HEK-293 cells and HEK-293 cells stably transfected with GPRC6A were prepared and stored at −80 °C. The membrane preparations were diluted to 0.15–0.5 mg of protein/ml in binding buffer (in mM: 20 HEPES, 100 NaCl, 6 MgCl2, 1 EDTA, and 1 EGTA) immediately before all binding assays. Total binding saturation curves were generated by incubating 250 μl of membrane preparation and 250 μl of [3H]testosterone ([1,2,6,7-3H]testosterone; 1 μCi; Sigma), dissolved in binding buffer (in mM: 20 HEPES, 100 NaCl, 6 MgCl2, 1 EDTA, and 1 EGTA) for final reaction concentrations ranging from 0.3 to 25 nM, for 40 min. After the 40-min incubation, binding reactions were terminated by rapidly filtering 400 μl of the reaction over a pre-soaked glass fiber filter (pore size, 1 μm, Whatman) to separate bound steroid from free steroid. The filters were immediately washed twice with 12.5 ml of wash buffer (PBS) and placed in a scintillation vial. Radioactivity was counted in a liquid scintillation counter (Beckman Instruments). All steps of the binding assays were conducted at 4 °C.

Testosterone Competition-binding Assays—Competitive receptor binding assays were performed (33) using [3H]testosterone to a final concentration of 40 nM and cold testosterone at concentrations of 0, 25, 50, 100, and 200 nM in HEK-293 cells stably transfected with GPRC6A (33). We performed the analysis by liquid scintillation counting using a liquid scintillation counter (Beckman Instruments).

Primary Bone Marrow Stromal Cells Culture—The femurs and tibias from 8-week-old wild type and GPRC6A−/− mice were dissected, the ends of the bones cut, and marrow flushed out with 2 ml of ice-cold α-minimal essential medium containing 10% FBS by using a needle and syringe. A suspension of bone marrow cells was obtained by repeated aspiration of the cell preparation through a 22-gauge needle, and nucleated cells were counted with a hemocytometer. Cells were seeded into 6-well plates at a density of 3 × 10⁷ cells/ml and cultured for 3 days in α-minimal essential medium supplemented with 10% FBS, 100 kilounits/liter of sodium penicillin G and 100 mg/liters of streptomycin sulfate in a humidified incubator with 5% CO₂ and 95% air at a temperature of 37 °C. On day 3, all nonadherent cells were then removed with the first medium change and then the adherent cells (representing bone marrow–derived mesenchymal stem cells, BMSCs) were grown for additional periods of up 3 days in the same medium. After overnight quiescence, the cells were stimulated for 5 min by testosterone and β-estradiol at the concentrations as indicated.

siRNA Suppression of GPRC6A Gene Expression—For GPRC6A knockdown experiments, a short interfering RNA (siRNA) (19 nucleotides hGPRC6A siRNA-202: CCAGAGTTGTTGGCTTTT) was designed by using the “siRNA target finder” at the Ambion website, from the hGPRC6A sequence (NM_148963). This siRNA hairpin was synthesized and cloned into a pSilencer™ 4.1-CMV neo vector (Ambion). The inserts were confirmed by direct sequencing. A circular pSilencer™ 4.1-CMV neo vector that expresses a hairpin siRNA with limited homology to any known sequence was used as a negative control (pSilencer™ 4.1-CMV neo Negative Control plasmid, supplied with the kit, Ambion). The constructs of siRNA duplexes were stably transfected into human prostate cancer cells using Lipofectamine™ (Invitrogen) and selected by G418 (Invitrogen). Successful knockdown of GPRC6A was confirmed by assessing RT-PCR analysis of GPRC6A expression.

Orchidectomy and Testosterone Replacement—Two-month-old male wild-type and GPRC6A null littermates were orchidectomized or sham operated and a slow-releasing pellet (5 mg/21 days, Innovative Research of America) containing either a placebo or testosterone was implanted subcutaneously 1 week after orchidectomy.

Non-genomic Effects of Testosterone in Vivo—Testosterone (200 mg/kg) or PBS vehicle was injected into the intraperitoneal cavity of wild-type GPRC6A+/+ and GPRC6A−/− mice and bone marrow was harvested at 20 and 60 min for assessment of ERK phosphorylation and Egr-1 mRNA expression (34). For serum LH and follicle-stimulating hormone (FSH) measurement, serum was collected after administration of testosterone by intraperitoneal injection. The serum LH and FSH were measured by mouse LH sandwich assay and mouse FSH radioimmunoassay as described by ligand assay and analysis at the core laboratory of the University of Virginia Health Science Center. This dose of testosterone was designed to achieve supraphysiological levels of circulating testosterone.

Statistics—We evaluated differences between groups by one-way analysis of variance. All values are expressed as mean ± S.E. All computations were performed using the Statgraphic statistical graphics system (STSC Inc.).

RESULTS

GPRC6A Responds to Extracellular Androgen and Other Steroids with Different Apparent Potencies in Vitro

To test the possibility that GPRC6A mediates the non-genomic effects of androgens, we initially assessed the ability of testosterone and the synthetic androgen R1881 to stimulate HEK-293 transfected with the full-length human GPRC6A
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FIGURE 1. Evidence for GPRC6A sensing extracellular androgens. A, RT-PCR analysis showing that GPRC6A and AR transcripts are endogenously expressed in 22Rv1 cells, but not in untransfected HEK-293 cells. HEK-293 cells stably transfected with a GPRC6A expression construct display abundant levels of GPRC6A message. Glyceraldehyde-3-phosphate dehydrogenase (G3PDH) was used as a control for RNA abundance. Data are representative of at least three independent experiments. B, dose-dependent effects of testosterone and R1881 to stimulate GPRC6A. Both testosterone and R1881 stimulated ERK activation in HEK-293 cells transfected with GPRC6A cDNA (upper panel), whereas non-transfected HEK-293 cells (middle panel) and HEK-293 cells transfected with CaSR cDNA, which encodes a related GPCR (lower panel), showed no response to exogenously added androgen ligands. C, time course of R1881 activation of ERK phosphorylation. D, extracellular calcium is required for GPRC6A sensing testosterone. At medium calcium concentrations of 0.5 mM, testosterone failed to activate GPRC6A. E, GPRC6A is activated by extracellular testosterone. The cell impermeable testosterone/BSA dose-dependently stimulated GPRC6A-mediated activation of phospho-ERK in HEK-293 cells transfected with GPRC6A (right panel) but not in untransfected HEK-293 cells (left panel). F, effects of testosterone/BSA in 22Rv1 human prostate cancer cells. 22Rv1 cells expressing GPRC6A endogenously also exhibited a dose-dependent stimulation of ERK in response to testosterone/BSA. Representative blots are shown, and the results were verified in at least three independent experiments.

We initially examined the effect of testosterone and R1881 to activate GPRC6A using ERK activity, which is a commonly used readout for assessing non-genomic actions of androgens in a variety of cell lines (10, 11, 36). We found that both testosterone and R1881 dose-dependently stimulated ERK activity in HEK-293 cells transfected with GPRC6A, but not in the non-transfected HEK-293 controls (Fig. 1B). The minimal concentration of R1881 and testosterone required to activate GPRC6A was ~20 nM. To test for receptor specificity, we examined the ability of the calcium sensing receptor (CaSR), a related family C GPCR, to respond to androgens. HEK-293 cells transfected with CaSR cDNA failed to respond to either extracellular testosterone or R1881 stimulation as assessed by phospho-ERK activity (Fig. 1B). The peak response to R1881 was observed at 10 min (Fig. 1C).

Allosteric modulators of this class of GPCRs require the presence of ligands, such as calcium, for receptor activation (37). We found that testosterone activation of GPRC6A also required calcium concentrations in excess of 0.5 mM (Fig. 1D), a concentration similar to the calcium requirement for amino acids and osteocalcin activation of GPRC6A (22, 38).

To explore if testosterone stimulation of GPRC6A is mediated by extracellular testosterone, we examined the effects of a BSA-testosterone conjugate, which cannot penetrate the cell. Testosterone/BSA (fresh dextran and charcoal treated) induced a dose-dependent stimulation of phospho-ERK in GPRC6A expressing HEK-293 cells (Fig. 1E, left panel), consistent with activation of a cell surface receptor. As control, testosterone/BSA did not stimulate phospho-ERK in untransfected HEK-293 cells (Fig. 1E, right panel). We also tested cell impermeable testosterone/BSA in human prostate cancer cells, 22Rv1, that expresses endogenous GPRC6A (Fig. 1F). Testosterone/BSA also induced a non-genomic response in 22Rv1 (Fig. 1F).

To examine ligand specificity, we examined several other steroids, including dehydroandrosterone, dihydrotestosterone, 17β-estradiol, and progesterone for their ability to activate GPRC6A. Dehydroandrosterone, dihydrotestosterone, and 17β-estradiol were able to elicit variable degrees of GPRC6A-mediated phosphorylation of ERK in HEK-293 cells transfected with GPRC6A, albeit at higher concentrations than testosterone (supplemental Fig. S1). In contrast, proges-
terone did not activate GPRC6A and appeared to inhibit ERK at concentrations up to 80 nM.

Receptor activation and potential cross-talk with AR signaling was also assessed in HEK-293 cells cotransfected with GPRC6A and AR cDNAs and either SRE-luciferase (SRE: serum response element; as a measure of G-protein receptor activation) or ARE-luciferase (ARE: androgen response element; as a measure of androgen receptor activation) reporter gene constructs (22, 39). R1881 dose-dependently stimulated SRE-dependent luciferase activity in GPRC6A expressing HEK-293 cells, but had no effect in HEK-293 control cells (Fig. 2A). Similarly, in HEK-293 cells co-transfected with GPRC6A and AR, calcium resulted in a dose-dependent activation of GPRC6A, and this response was not influenced by the co-expression of AR (Fig. 2B). To further exclude a role of AR in GPRC6A signaling, we examined the effect of the non-steroidal anti-androgens, flutamide and bicalutamide, on testosterone-stimulated ERK activity. Flutamide and bicalutamide had no effect on testosterone-stimulated GPRC6A activation of phospho-ERK (supplemental Fig. S2). Next, we examined if GPRC6A modulates the genomic effects of AR. HEK-293 cells transfected with AR and/or GPRC6A were stimulated with R1881 and AR-mediated transcription was assessed by measuring ARE-luciferase activity (Fig. 2C). R1881 failed to stimulate ARE-luciferase activity in HEK-293 cells expressing only GPRC6A (Fig. 2C), but stimulated ARE-luciferase activity in HEK-293 cells transfected with AR, consistent with activation of nuclear receptor signaling (Fig. 2C). In HEK-293 cells co-expressing both GPRC6A and AR, however, R1881-mediated activation of ARE-luciferase activity was significantly attenuated (Fig. 2C), suggesting potential cross-talk between GPRC6A and AR pathways.

**Androgens Binding to GPRC6A**

We previously demonstrated that GPRC6A overexpression results in cell surface expression of this receptor (22–23). To further confirm that androgen binding is increased in the cell surface of GPRC6A expressing cells, we established that GPRC6A overexpression resulted in androgen binding sites on the surface of HEK-293 cells, we incubated cells transfected with GPRC6A cDNA constructs with testosterone/BSA conjugated to FITC. After incubation for 5–10 s, testosterone/BSA/FITC was detected on the surface of HEK-293 cells transfected with GPRC6A, but not in empty HEK-293 cells (Fig. 3A). As control, we also found that BSA/FITC did not bind to the cell surface of HEK-293 cells transfected with GPRC6A (Fig. 3A). In addition, we isolated the membrane fractions from the HEK-293 cells, stably transfected GPRC6A and control HEK-293 cells. Significant amounts of specific [3H]testosterone binding were detected in plasma membranes of the HEK-293 cells transfected with GPRC6A, whereas negligible specific binding was detected in the plasma membranes of untransfected cells (Fig. 3B). To further elucidate that GPRC6A specifically binds to testosterone, we performed a steroid testosterone competition-binding assay. Steroid testosterone competition study showed that binding is specific for testosterone (Fig. 3C). These data suggest that GPRC6A imparts cell surface binding of testosterone.

To provide a preliminary hypothesis regarding the residues responsible for testosterone recognition, homology models of GPRC6A were constructed. Testosterone was successfully docked under EL2 in both models, and common hydrophobic residues from EL2 were observed to surround the hydrophobic ring system of testosterone in both models (supplemental Fig. S3). These data suggest that further
modeling and experimental studies are warranted that define a more accurate structure of EL2 and investigates the role of hydrophobic residues in that loop, such as Val-734, in testosterone binding.

GPRC6A-coupled Signal Transduction Pathways and Downstream Targets

Existing evidence implicates a pertussis toxin-sensitive GPCR in the non-genomic effects of androgens (40) and we and others have previously shown that GPRC6A can couple to a pertussis toxin-sensitive \( \alpha \) subunit, activation of ERK signaling pathways (22, 38). Consistent with these observations, we found that testosterone activation of phospho-ERK was significantly blocked by 100 ng/ml of pertussis toxin (PTx) (Fig. 4A). We also found that testosterone-stimulated GPRC6A-mediated phospho-ERK or SRE-luciferase activity were blocked by PD89059 (MAPK inhibitor), Ly294002 (PI3K inhibitor), and PP-1 (Src inhibitor) (left panel). The inhibitors of PD89059, Ly294002, and PP-1 had nonspecific effects on activities of phospho-ERK in HEK-293 cells stably transfected with GPRC6A without testosterone stimulation (right panel). C, R1881 stimulated GPRC6A activation of SRE-luciferase was also inhibited by PD89059, the PKC inhibitor Ro31-8220, and Src inhibitor PP-1. HEK-293 cells were co-transfected with pcDNA3.GPRC6A and SRE-luciferase reporter gene plasmid. The values depicted represent the mean ± S.E. of luciferase activity in a minimum of three separate experiments. D and E, time course of R1881 activation of Raf-1 and p-Src. R1881 stimulated GPRC6A-mediated non-genomic activation of intercellular phospho-Raf-1 (D) and phospho-Src (E).
hibitor), and Ro31–8220 (PKC inhibitor), but the inhibitors alone did not affect the basal ERK level (Fig. 4, B and C). In addition, R1881 also stimulated GPRC6A-mediated activation of phospho-Raf-1 (Fig. 4D, upper panel) and phospho-Src (Fig. 4E) in HEK-293 cells transfected with GPRC6A. These results suggest that Gαq, PI3K, PKC, Src, and Ras/Raf/ERK may be involved in the testosterone-stimulated GPRC6A-mediated signaling pathway.

**Demonstration of GPRC6A Androgen Sensing Function in ex Vivo Cell Culture Systems Expressing Endogenous GPRC6A**

The non-genomic effects of androgens are present in many cell types, including osteoblasts and bone marrow stromal cells (9, 11, 17, 41, 42). By reverse transcriptase-mediated polymerase chain reaction (RT-PCR) we found that GPRC6A transcripts were present in bone marrow and testis in wild-type mice, but not in GPRC6A−/− mice (Fig. 5A). To evaluate the androgen sensing potential of GPRC6A in cells *ex vivo*, we compared the ability of BMSC obtained from wild-type and GPRC6A−/− mice to respond to testosterone added to the culture medium (Fig. 5B). We observed that testosterone at concentrations up to 80 nM had minimal effects to stimulate phospho-ERK activity in BMSC from GPRC6A−/− mice compared with its substantial stimulation of ERK in cells from wild-type littermates (Fig. 5B). We also tested two other ligands for GPRC6A, extracellular calcium and the calcimimetic NPS-R568 (25). Consistent with the loss of GPRC6A, BMSC from GPRC6A−/− mice failed to respond to either extracellular calcium or the calcimimetic NPS-R568, whereas BMSC from WT mice exhibited both extracellular calcium- and NPS-R568-dependent stimulation of ERK phosphorylation (43).

We also examined whether the non-genomic response to extracellular androgen could be blocked by siRNA directed against GPRC6A in human prostate cancer 22Rv1 cells. GPRC6A mRNA was reduced in 22Rv1 cells transfected with the plasmid encoding hairpin siRNAs against GPRC6A (hGPRC6A siRNA-202 or hGPRC6A siRNA-514) as compared with cells transfected with a plasmid encoding a negative control siRNA (Fig. 5C). The activation of phospho-ERK stimulated by GPRC6A ligands, extracellular calcium, and testosterone in the 22Rv1 human prostate cancer cell was also significantly decreased by transfected hGPRC6A siRNA-202 or hGPRC6A siRNA-514 (Fig. 5D). In addition, we examined

**FIGURE 5. GPRC6A mediates the non-genomic effects of androgens *ex vivo*.** A, GPRC6A is expressed in testis and bone marrow derived from wild-type mice but not in GPRC6A−/− mice by RT-PCR. B, BMSCs derived from the male GPRC6A−/− mice exhibited reduced ability to activate ERK in response to testosterone (80 nM). C, overexpression of hGPRC6A siRNA-202 and siRNA-514 inhibited GPRC6A mRNA expression in 22Rv1 cells. D and E, effect of GPRC6A knockdown in 22Rv1 prostate cancer cells. siRNAs mediated knockdown of GPRC6A inhibited testosterone, calcium and osteocalcin stimulated phospho-ERK activation (D) and cell proliferation (E) in 22Rv1 cells. G6PDH, glyceraldehyde-3-phosphate dehydrogenase.
whether the cell proliferation induced by extracellular testosterone could be blocked by siRNA directed against GPRC6A. hGPRC6A.siRNA-202 or hGPRC6A.siRNA-514, but not negative control siRNA blocked cell proliferation induced by extracellular testosterone (Fig. 5E, left panel). We also found that other GPRC6A ligands, calcium and osteocalcin-induced cell proliferation were inhibited by transfecting hGPRC6A.siRNA-202 into 22Rv1 cells (Fig. 5D, middle and right panels).

**In Vivo Evidence for GPRC6A Mediation of Non-genomic Effects of Testosterone**

**Resistance to Non-genomic Effects of Androgens in Organs from GPRC6A$^{-/-}$ Mice in Vivo**—To establish a link between non-genomic effects of androgens and tissue responses in vivo, we examined the impact of loss of GPRC6A on the capacity of testosterone to stimulate phospho-ERK activity and early growth responsive 1 (Egr-1) expression in bone marrow and testes isolated from WT and GPRC6A$^{-/-}$ mice (Fig. 6) after the intraperitoneal administration of testosterone. We found that testosterone treatment at a pharmacological dose of 200 mg/kg stimulated both ERK activity (Fig. 6, A and B) and Egr-1 expression (Fig. 6, C and D) in bone marrow and testes of wild-type mice, but this response was markedly attenuated in GPRC6A$^{-/-}$ mice.

Seminal vesicle size is a sensitive measure of androgen genomic effects. Seminal vesicle size is reduced in androgen receptor mutant mice (25, 44, 45). Because GPRC6A deficiency also causes alterations of serum testosterone levels (25), it is possible that reduced testosterone levels might also contribute to the observed phenotype in GPRC6A$^{-/-}$ mice via the classical androgen-dependent mechanism (45, 46).

First, we examined GPRC6A expression in the seminal vesicle by RT-PCR, and found that GPRC6A transcripts were present in seminal vesicle from wild-type mice, but not in GPRC6A$^{-/-}$ mice (Fig. 7A). To explore the relative role of genomic actions and non-genomic actions of testosterone, we orchietomized male wild-type and GPRC6A$^{-/-}$ littermates followed by administration of testosterone by implanting slow release drug eluting pellets. The size of seminal vesicles from GPRC6A$^{-/-}$ mice was slightly reduced compared with wild-type mouse orchietomy resulted in significant reductions in the size of the seminal vesicles in both wild-type and GPRC6A$^{-/-}$ littermates compared with uncastrated controls (Fig. 7B, middle panel). Testosterone administration resulted in full restoration of the size of seminal vesicles in orchietomized wild-type mice (about 9-fold increased), but the recovery in orchietomized GPRC6A$^{-/-}$ mice was significantly less (Fig. 7B, lower panel), the weight of seminal vesicles in orchietomized GPRC6A$^{-/-}$ mice recovered but testosterone administration was less (about 6-fold increased) than wild-type mice (Fig. 7C). These results are consistent with a functional role of GPRC6A in regulating the response to androgens in seminal vesicles, either through direct sensing of androgens by GPRC6A or indirect effects of GPRC6A to cross-talk with AR function.

**Paradoxical Stimulation of LH by Testosterone in GPRC6A Null Mice**—A better marker of non-genomic actions of testosterone is its effects to regulate LH release, which occurs through more classical GPCR kinetics. Serum LH levels are negatively regulated by testosterone-mediated effects on gonadotropin releasing hormone and by direct effects on LH secretion by the pituitary (47). Given the known role of GPCR in mediating the rapid changes in hormone secretion in many regulatory networks, we examined if there is

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**FIGURE 6. Resistance to non-genomic effects of androgens in organs from GPRC6A$^{-/-}$ mice.** Testosterone (200 mg/kg) or PBS vehicle was injected intraperitoneally into wild-type and GPRC6A$^{-/-}$ male mice. A and B, Western blot analysis of ERK phosphorylation in bone marrow (A) and testis (B) 20 min after injection of testosterone shows loss of testosterone-mediated ERK activation in GPRC6A null tissues. C and D, RT-PCR analysis of the Egr-1 mRNA abundance in bone marrow (C) and testis (D) 60 min after intraperitoneal injection of testosterone also shows loss of testosterone-mediated activation in GPRC6A null tissues. G6PDH, glyceraldehyde-3-phosphate dehydrogenase.
evidence for a non-genomic function of GPRC6A in regulating LH secretion. To investigate any abnormality of the hypothalamic-pituitary-gonadal axis in GPRC6A−/− mice, we administrated 200 mg/kg of testosterone to the wild-type and GPRC6A−/− mice and measured serum testosterone, LH, and FSH after 2 h administration. We found that serum testosterone increased from 2 to 150 nM 2 h after injection. The serum LH level was decreased after administration of testosterone in wild-type mice (Fig. 7D, left panel). In GPRC6A−/− mice, the serum LH level was higher than wild-type mice as previously reported (25), but was paradoxically further increased (5-fold) after administered testosterone (Fig. 7D, left panel). The serum FSH was not changed before or after administration of testosterone between wild type and GPRC6A−/− mice (Fig. 7D, right panel).

DISCUSSION

Several mechanisms have been proposed to explain the rapid cellular responses to steroids (5, 9), such as translocation of steroid nuclear receptors to the cell surface membrane (12–14), nonspecific effects of steroids on the fluidity of lipids in the plasma membrane, direct allosteric modification of ligand-gated ion channels (5, 48, 49), and GPCRs (9, 15, 16, 50, 51). GPRC6A is an amino acid, calcium, and osteocalcin sensing G-protein coupled receptor that appears to coordinate anabolic responses in multiple tissues (25). In the current investigations, we found that anabolic steroids, including testosterone, can activate GPRC6A, suggesting that GPRC6A may mediate the non-genomic effects of testosterone and other anabolic steroids.

The role of GPRC6A in mediating the non-genomic actions of anabolic steroids is supported by several findings. First, in
vitro studies in heterologous cell culture models demonstrated the androgen-sensing functions of GPRC6A. Indeed, GPRC6A rapidly stimulated both ERK activation and SRE-promoter-luciferase activity in response to testosterone in a GPRC6A-dependent fashion. This rapid response was not dependent on endogenous AR, because the experimental cell models lacked the AR and the anti-androgen flutamide and bicalutamide failed to inhibit the response. In addition, we found that impermeable testosterone/BSA activated ERK in cells transfected with GPRC6A but not in untransfected control cells. Second, cells that express endogenous GPRC6A lost the rapid signaling responses to androgens after deletion or knockdown of this receptor. In this regard, BMSC derived from GPRC6A−/− mice displayed less testosterone-stimulated ERK activity compared with wild-type mice. We also found that 22Rv1 prostate cancer cells expressed GPRC6A and displayed rapid signaling responses to testosterone that were inhibited by siRNA-mediated knockdown of GPRC6A. Third, the in vivo relevance of this response was established by identifying several rapid responses that were attenuated in GPRC6A null mice. Indeed, we found that testosterone-stimulated ERK activity and Egr-1 expression in bone marrow and testis were significantly less in GPRC6A−/− compared with wild-type mice. In addition, testosterone injections paradoxically increased serum LH levels in GPRC6A−/− mice, but suppressed LH in wild-type mice.

To our knowledge GPRC6A is the first nonclassical receptor for which androgen responses have been demonstrated both in vitro and in vivo. Our findings may have biological relevance with regard to understanding the side effects of the pharmacological misuse of anabolic steroids in sports and body building. At present, however, because we used pharmacological dosing of testosterone in vivo, and have not yet defined the specific gene networks regulated by non-genomic effects of testosterone, the physiological relevance of our findings remain to be established.

Multiple signal transduction pathways have been linked to the non-genomic response to androgens, including adenyl cyclase, mitogen-activated protein kinases (MAPKs), phosphatidylinositol 3-kinase (PI3K), and intracellular calcium concentrations (36, 52). GPRC6A is purported to be coupled to Gαs and Gαq, but evidence demonstrating activation of intracellular calcium and inhibition of cAMP are variable and inconsistent and the most reliable readout for GPRC6A activation has been ERK and SRE-luciferase promoter-reporter activity (22, 38). In the current study, we used these readouts and biochemical inhibitors to demonstrate that Gαs, PI3K, PKC, Src, and Ras/Raf/MEK/ERK pathways are downstream effectors of GPRC6A. These results resemble non-genomic responses to testosterone observed in macrophages and osteoblasts that also involve activation of PI3K, PKC, c-Src, c-Raf-1, and MEK1/2 via a putative pertussis toxin-sensitive G-protein (53, 54). Our findings, however, do not exclude the existence of other molecular targets or other signal transduction pathways that may also mediate the non-genomic actions of sex steroids. Indeed, several other GPCRs are reported to mediate the non-genomic effects of androgens in vitro (55).

Many tissues are purported to have non-genomic responses to androgens, including endothelial cells, osteoblasts, skeletal muscle cells, synaptosomes, prostate cells, T cells, and macrophages (17, 54, 56, 57). Non-genomic effects of GPCRs have been implicated in a variety of physiological processes, including anesthetic and antiepileptic actions, changes in neuronal activity, neurodegenerative diseases, facilitation of the sperm acrosome reaction, oocyte maturation, insulin sensitivity in adipocytes, endothelial dysfunction, and vasodilation (58–63), but none of these have been confirmed by loss-of-function of the non-genomic molecular target. Our studies provide insights, for the first time, into the precise non-genomic actions of testosterone, beyond that of modulating earlier signaling responses by assessing non-genomic tissue response in the presence and absence of GPRC6A. In this regard, we discovered that loss of GPRC6A results in a paradoxical stimulation of LH, indicating a non-genomic function of GPRC6A to regulate the hypothalamus-pituitary axis, a pathway that is consistent with a GPCR-regulated tissues. Further investigations of GPRC6A regulation of gonadotropin releasing hormone and LH secretion will be of great interest. We also found evidence that GPRC6A may be important in regulation of prostate cell proliferation, because extracellular testosterone, calcium, and osteocalcin stimulate 22Rv1 prostate cancer cell proliferation in a GPRC6A-dependent fashion. It is of potential interest that GPRC6A senses osteocalcin, a bone derived factor that could be involved in homing of prostate cancer cells to bone via GPRC6A or other mechanisms (64). Our data suggests that GPRC6A may participate in the integrative physiology of multiple tissues in response to anabolic steroids as well as alterations in cations, and amino acid nutrients and bone-derived osteocalcin. Regardless, additional studies that assess the dose-dependent effects of testosterone on multiple organs in GPRC6A null, AR null, and combined GPRC6A and AR null mice will be needed to separate the physiologically relevant non-genomic and genomic actions of testosterone.

The response to chronic administration of testosterone was also altered in GPRC6A−/− mice. In this regard, exogenously administered testosterone only partially restored the seminal vesicle weight and size in orchectomized GPRC6A−/− mice at doses that fully restored these organs in orchectomized wild-type controls. This suggests possible cross-talk between the non-genomic effects of GPRC6A and the AR-mediated responses. We have evidence for GPRC6A to modulate both the metabolism of testosterone and the transcriptional activity of AR. Loss of GPRC6A also leads to reductions in CYP 17, the enzyme that converts testosterone to dihydrotestosterone (supplemental Fig. S4), and cotransfection of GPRC6A and AR reduces AR-mediated gene transcription in response to testosterone. There are precedents in other biological systems for coordination between a GPCR and steroid receptor, such as CaSR and VDR regulation of parathyroid gland function (65). Typically the GPCR regulates acute changes and the steroid receptor leads to more long-term regulation of gene expression. Similarly, because testosterone levels can change acutely in response to a variety of stimuli (66, 67), GPRC6A may provide a mechanism to respond to short-term variations.
in circulating androgen levels and may work in concert with the AR in more chronic responses. Regardless, the new biological insight from these studies is that GPRC6A-mediated non-genomic responses and AR-dependent genomic responses can have coordinated functional effects in androgen responsive tissues. Further studies are needed to define the common and differential gene regulation and the temporal sequence of these GPRC6A- and AR-dependent effects.

There are several potential limitations to our study. First, although GPRC6A activation by testosterone was dose-dependent (10–100 nM), the concentrations of testosterone required to activate this receptor in vitro was somewhat higher than the dose required for activation of AR (68, 69) and to stimulate non-genomic effects in osteoblasts (11), T cells, and macrophages (70), but comparable with the testosterone dose required to activate the non-genomic response in skeletal muscle cells (71). Second, we found that the in vitro response of GPRC6A was not specific for testosterone. Indeed, estrogen, albeit at higher concentrations, but not progesterone, were able to activate GPRC6A in heterologous cell systems. In addition, binding kinetic studies that calculate $B_{\text{max}}$ and $K_d$ for testosterone binding to GPRC6A and validation of our preliminary computational models defining the testosterone binding domains are needed. We also did not fully define the differential effects of genomic actions mediated by GPRC6A and genomic actions mediated by AR. A better understanding of the potential interdependent and separate biological effects of testosterone activation of GPRC6A and androgen receptors will require further study. Comparative analysis of testosterone responses in GPRC6A null, AR null, and combined GPRC6A and AR null mice may provide insights into the inter-relationships between non-genomic effects mediated by GPRC6A and genomic actions of androgens mediated by AR (45, 46). Regardless, androgens have beneficial effects on bone and muscle mass and other tissues, but their negative impact on the prostate prevents their widespread use in the treatment of osteoporosis and frailty in males (72). Development of selective androgen receptor modulators that activate AR but not GPRC6A and vice versa might be useful in obtaining greater therapeutic specificity.

In conclusion, GPRC6A is widely expressed in many tissues (22, 23, 73) and its ablation results in loss of testosterone-dependent rapid signaling in target tissues, consistent with a physiological role of this receptor in mediating non-genomic androgen responses. GPRC6A is also activated by extracellular calcium, calcimimetics, amino acids, and osteocalcin (22, 23, 73), the latter also functions as a hormone regulating energy metabolism (74). These sensing properties of GPRC6A, along with its responsiveness to anabolic steroids, may provide a means to coordinate nutritional and hormonal anabolic signals in multiple organs. As such, GPRC6A is a potential target for developing antagonists and agonists that would have broad applications in health and disease.

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