Reproductive Biology and Endocrinology

Research

4-Aminopyridine Decreases Progesterone Production by Porcine Granulosa Cells

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Abstract

Background: Ion channels occur as large families of related genes with cell-specific expression patterns. Granulosa cells have been shown to express voltage-gated potassium channels from more than one family. The purpose of this study was to determine the effects of 4-aminopyridine (4-AP), an antagonist of KCNA but not KCNQ channels.

Methods: Granulosa cells were isolated from pig follicles and cultured with 4-AP, alone or in combination with FSH, 8-CPT-cAMP, estradiol 17β, and DIDS. Complimentary experiments determined the effects of 4-AP on the spontaneously established pig granulosa cell line PGC-2. Granulosa cell or PGC-2 function was assessed by radio-immunoassay of media progesterone accumulation. Cell viability was assessed by trypan blue exclusion. Drug-induced changes in cell membrane potential and intracellular potassium concentration were documented by spectrophotometric determination of $DiBAC_4(3)$ and PBFI fluorescence, respectively. Expression of proliferating cell nuclear antigen (PCNA) and steroidogenic acute regulatory protein (StAR) was assessed by immunoblotting. Flow cytometry was also used to examine granulosa cell viability and size.

Results: 4-AP (2 mM) decreased progesterone accumulation in the media of serum-supplemented and serum-free granulosa cultures, but inhibited cell proliferation only under serum-free conditions. 4-AP decreased the expression of StAR, the production of cAMP and the synthesis of estradiol by PGC-2. Addition of either 8-CPT-cAMP or estradiol 17β to serum-supplemented primary cultures reduced the inhibitory effects of 4-AP. 4-AP treatment was also associated with increased cell size, increased intracellular potassium concentration, and hyperpolarization of resting membrane potential. The drug-induced hyperpolarization of resting membrane potential was prevented either by decreasing extracellular chloride or by adding DIDS to the media. DIDS also prevented 4-AP inhibition of progesterone production.

Conclusion: 4-AP inhibits basal and FSH-stimulated progesterone production by pig granulosa cells via drug action at multiple interacting steps in the steroidogenic pathway. These inhibitory effects of 4-AP on steroidogenesis may reflect drug-induced changes in intracellular concentrations of $K^+$and Cl^- as well as granulosa cell resting membrane potential.

Background

Ion channels located in the plasma membrane provide one means to mediate cellular adaptation to local environmental changes. Voltage-gated K^+ channels in non-nerve, non-muscle cells play crucial roles in cell development, proliferation, migration, volume regulation, as well as maintenance of membrane potential and cell viability. This is in part because these channels regulate the cytoplasmic concentrations of K^+, Ca^{2+}, and Na^+ ions [1–10]. The diversity of voltage-gated K^+ channels and currents present in these "non-excitable" (non-muscle, non-nerve) cells is impressive. Thus, the assignment of specific functions to particular subclasses of K^+ channel proteins represents a significant challenge [4,10–16].

Voltage-gated K^+ currents with distinct electrophysiological and pharmacological properties are present in granulosa cells (GC), and modulate resting membrane potential [4,12,13,16,17]. Furthermore, selective antagonism of GC K^+ channels with distinct molecular correlates, electrophysiological properties and expression patterns can influence differentially GC proliferation, steroidogenic capability, and apoptosis [17,18]. Our laboratory has previously identified two distinct delayed rectifier K^+ currents in pig GC: a slow current (I_{Ks}) associated with channels formed by co-assembly of KCNQ1 pore-forming and KCNE1 accessory proteins, and an ultra-rapid current (I_{Kur}) formed by co-assembly of KCNA pore-forming and KCNAB accessory proteins [4]. Moreover, we have shown that selective block of I_{Ks} enhances basal progesterone synthesis, while complete block of both I_{Ks} and I_{Kur} accelerates apoptosis [18].

Here, our goal was to determine the functional effects of antagonizing GC K^+ channels formed by KCNA vs. KCNQ family proteins. To this end, we treated primary cultures of GC and monolayers of the immortalized granulosa cell line PGC-2 [19] with the K^+ channel antagonist 4-aminopyridine (4-AP). 4-aminopyridine, at millimolar concentrations, completely inhibits K^+ currents conducted by heterologously expressed KCNA channels and native GC I_{Kur}, but lacks significant effects on native GC I_{Ks} [4,12,13,20]. Kusaka showed that antagonism of 4-AP sensitive slowly inactivating delayed rectifier K^+ currents suppressed both basal and luteinizing hormone (LH)-stimulated progesterone production [21]. In this report, we extend those findings by demonstrating 4-AP inhibition of follicle stimulating hormone (FSH)-stimulated progesterone production, and by elucidating the underlying mechanisms.

Methods

Reagents

Cell culture media, supplements, phosphate-buffered saline (PBS, 10 X) and sera were obtained from Life Technologies (Gaithersburg, MD) unless stated otherwise. Chemicals were obtained from Sigma (St. Louis, MO) unless stated otherwise. Regular pork insulin (100 units/mL) was obtained from Eli Lilly (Indianapolis, IN). PBFI-AM (1,3-Benzenedicarboxylic acid, 4,4'-[1,4,10,13-tetraoxa-7,16-diazacyclooctadecane-7,16-diylbis(5-methoxy-6,2-benzofurandiyl)]bis-, tetrakis [(acetyloxy)methyl] ester) and $DiBAC_4(3)$ (bis-(1,3-dibutylbarbituric acid) trimethine oxonol) were obtained from Molecular Probes (Eugene, OR). Primary antibodies were obtained from commercial sources for detection of proliferating cell nuclear antigen (PCNA, Oncogene Science, Cambridge, MA) and actin (Sigma). Nitrocellulose membranes (Hybond ECL), secondary antibodies, enhanced chemiluminescence (ECL) reagent, and film (Hyperfilm ECL) were obtained from Amersham Pharmacia Biotech (Piscataway, NJ). Porcine FSH (2039 IU/mg) was obtained from the National Hormone and Pituitary Program (NHPP). PGC-2 cells were provided by B.R. Downey (McGill University, Montreal, Canada). Antibody directed against steroidogenic acute regulatory protein (StAR) was a gift from D.B. Hales (University of Illinois, Chicago).

GC Isolation and Culture

Porcine ovaries were collected at a local slaughterhouse, and GC were isolated using techniques described previously in detail [4,18]. Briefly, small (1–3 mm diameter) to medium (4–6 mm diameter) follicles were aspirated by hand using a 19-gauge needle attached to a 10 cc syringe. GC were separated from follicular fluid by centrifugation at 500 × g for 5 minutes. Cells were washed twice with a 1:1 mixture of Ham F10 nutrient medium and Dulbecco modified Eagle medium (DMEM) containing 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES, 25 mM), penicillin (50 U/ml) and streptomycin (50 μg/ml). Culture conditions for GC were similar to those described previously by our laboratory and others [4,18,22,23]. The basic culture medium consisted of HEPES-buffered DMEM: F10 (1:1) supplemented with fetal bovine serum (FBS, 10%), penicillin (50 U/ml), streptomycin (50 μg/ml), gentamicin (57 ng/ml) and amphotericin (2.5 μg/ml).

Freshly isolated GC were plated in the basic serum-supplemented culture media on collagen-coated 24-well (500 μl/well) or 6-well (2 ml/well) culture dishes, and incubated at 37° C in a humidified atmosphere of 5% CO_2 and air. In every experiment, cells were plated at equal density for each treatment group. Plating density between experiments varied from 2×10^4 cells/well/24-multiwell to 1×10^6 cells/well/6-multiwell. Culture wells in the 6-multiwell clusters had a well diameter of 34.8 mm and a growth area of 9.4 cm^2, while culture wells in the 24-multiwell clusters had a well diameter of 15.6 mm and a growth area of 1.9 cm^2. The culture media were changed 16 h after

plating to fresh serum-supplemented media that contained either vehicle or treatment(s). Media were collected and replaced at 24 h intervals thereafter. 4-AP was dissolved directly into the culture media on the day of use, and the pH was readjusted to 7.4 with saturated HCl after addition of drug. DIDS (4',4' diisothicyanato-stilbene-2-2'-disulfonic acid) was diluted into culture media from a 200X aqueous stock. Stock solutions of forskolin (10 mM) and 3-isobutyl-1-methylxanthine (IBMX, 80 mM) were in DMSO.

In a subset of experiments, GC were cultured in a defined serum-free medium of HEPES-buffered DMEM:F10 (1:1) containing insulin (300 mU/ml), hydrocortisone (40 ng/ml), transferrin (5 μg/ml), bovine serum albumin (4 mg/ml, Bovine Albumin Fraction V, albumin = 95%), gentamicin (57 μg/ml), penicillin (50 U/ml), streptomycin (50 μg/ml), and amphotericin (2.5 μg/ml). This medium significantly increases the magnitude and duration of basal and FSH-stimulated progesterone output by GC [22].

PGC-2 Cell Culture and Patch Clamp

The pig granulosa cell line PGC-2 was obtained from Bruce Downey (McGill University) and maintained in culture in McCoy's modified 5A medium supplemented with 10% FBS, as described previously in detail [19]. PGC-2 cells have been well-characterized with respect to steroidogenic capability, cAMP response, and gonadotropin insensitivity [19]. Here, PGC-2 were seeded at a density of approximately 5×10^5 cells/ml of serum-containing medium in 24-well tissue culture plates. After 24 h the medium was replaced with serum-free medium containing either pregnenolone (1 μg/ml), androstenedione (100 μM), 5α-dihydrotestosterone (5α-DHT, 10 or 100 μM), or forskolin (5 μM) and IBMX (10 μM). To ensure the validity of this model for assessing the functional effects on the K^+ channel antagonists, we used whole-cell patch clamp techniques [4] to document the presence of 4-AP sensitive K^+ currents in PGC-2 (see additional file 1).

Measurement of Membrane Potential

The resting membrane potentials of cultured GC were assessed by spectrophotometric determination of $DiBAC_4(3)$ fluorescence (excitation= 485 nM; emission= 527 nM; Fluoroskan Ascent FL, LabSystems Inc., Helsinki, Finland). Cells were equilibrated with 2 μM $DiBAC_4(3)$ for 30 minutes at 37°C prior to fluorescence measurement. In most experiments, GC were bathed in culture media. However, one set of fluorescence measurements compared GC incubated in a normal Tyrode solution containing (in mM): 132 NaCl; 4 KCl; 1 $MgCl_2$; 1 $CaCl_2$; 5 dextrose; 10 HEPES (pH = 7.4) to GC similarly incubated in a relatively low-chloride Tyrode solution in which the NaCl was replaced with sodium aspartate.

Measurement of Intracellular Potassium

Intracellular levels of potassium were assessed by determination of PBFI fluorescence. Cells were equilibrated with the cell permeant acetoxylmethyl ester of the ion sensitive dye PBFI (5 μM) for 1 h, then washed and resuspended in PBS. Fluorescence was measured using a plate-reader (excitation = 340 nM; emission = 505 nM; Fluoroskan Ascent FL, LabSystems Inc., Helsinki, Finland).

Preparation of GC Lysates

Whole cell lysates were made from GC monolayers by standard techniques using a lysis buffer consisting of PBS with 1% Nonidet P40, 0.5% sodium deoxycholate, 0.1% SDS and protease inhibitor cocktail (1:100, Sigma P8340). Lysis buffer was added to the culture dish after washing with cold PBS 3 times. The culture dishes were scraped and the lysate was aspirated into a syringe with a 21-gauge needle to shear DNA. The lysates were rocked in the cold for 1 h and centrifuged for 10 min at 10,000 × *g*. In some cases, a highly enriched mitochondrial fraction was obtained using a commercially available kit (Mitchondrial Fractionation Kit, Active Motif, Carlsbad, CA) according to the manufacturer's instructions. Protein concentrations of GC lysates were determined by the bicinchoninic acid method (Micro BCA Protein Assay, Pierce, Rockford IL).

Immunoblotting

For direct comparisons of protein expression between drug-treated and untreated cells, equal amounts of protein were loaded in adjacent lanes on a single polyacrylamide gel, separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions and transferred to nitrocellulose membranes by the semi-dry transfer method. The membranes were blocked for 1 h at room temperature with 5% nonfat milk in Tris-buffered saline (TBS: 100 mM Tris, 0.9% NaCl, pH 7.5) containing 0.1% Tween 20, then incubated overnight at 4°C with primary antibody diluted in the blocking solution. Primary antibody dilutions were: PCNA (1:200); actin (1:500); StAR (1:500). After three washes with 0.1% Tween/TBS, the membranes were incubated for 1 h at room temperature with the appropriate horseradish peroxidase conjugated secondary antibody diluted 1:1500 in 5% nonfat milk/0.1% Tween/TBS. After four additional washes with 0.1% Tween/TBS, bound primary antibodies were visualized using an ECL detection system and recorded on radiographic film (Amersham Pharmacia Biotech). Densitometric analysis was performed using Scion Image (Scion Corporation, Frederick, MD). Equal loading was confirmed by either immunoblotting for actin or Coomassie-blue staining of gel lanes loaded and subjected to SDS-PAGE in a manner identical to those used for immunoblotting.

Immunoassays

Progesterone concentrations were determined by a solid-phase radioimmunoassay (CAC Progesterone, Diagnostic Products Corp, Los Angeles, CA) validated for measuring progesterone in the culture media. The assay sensitivity was approximately 0.3 ng/ml. The cross-reactivity with androstenedione (1000 ng/mL) was 0.047%. The cross-reactivity with pregnenolone (500 ng/mL) was 0.30%. The within and between assay coefficients of variation for the progesterone assay were 4.3 and 11.7%, respectively.

Aliquots of culture media were stored at -20°C for up to 60 days prior to progesterone assay. In the initial series of experiments where serial progesterone measurements were made from a single culture well, media progesterone concentrations 24–48 hours post-treatment were normalized to pre-treatment concentrations. In subsequent experiments, progesterone concentrations at various time-points post-treatment were normalized to either cell number or protein concentration.

Estradiol-17β was measured using commercially available reagents (TKE2, Diagnostic Products Corp, Los Angeles, CA). This assay had been validated previously for porcine serum [24], and was further modified and validated for use with culture media containing FBS. In brief, estradiol-17β could be quantitatively recovered when added to culture media, and increasing dilutions of media spiked with estradiol-17β produced similar concentrations when evaluated in the assay. The sensitivity was 4.9 pg/ml. The intra- and interassay coefficients of variation for estradiol assay were 4.8 and 8.3%, respectively.

Cyclic AMP was measured in GC lysates using an enzyme immunoassay according to the instructions provided by the manufacturer (Assay Designs, Ann Arbor, MI).

Determination of Cell Number

The number of viable GC in the monolayer cultures was determined by first harvesting the attached cells with a trypsin-containing solution (mg/ml): NaCl 8; KCl 0.4; dextrose 1; $NaHCO_3$ 0.6; ethylenediaminetetraacetic acid sodium salt (Na-EDTA) 0.2; trypsin 0.5, and then counting directly the number of viable cells using trypan blue exclusion and hemacytometry.

Flow Cytometry

Flow cytometry was also used to assess GC viability and size, using methods described previously in detail to determine propidium iodide uptake and forward (small angle light) scatter, respectively [18,25]. Briefly, ten thousand cells were examined per sample using a Becton Dickinson FACSCalibur to excite the cells with a 488 nM argon laser, and the collected data obtained were analyzed using Cell Quest™ software (Becton Dickinson, Mount View, CA). Changes in the forward light scattering properties of GC were used to infer changes in GC size. Debris, shrunken (apoptotic) GC and dead GC stained by propidium iodide were excluded from analysis.

Statistical Analysis

Data are expressed as mean ± SEM unless stated otherwise. Statistical analysis of treatment effects on forward scatter characteristics was performed using the non-parametric Friedman test. For all other data, significant differences between groups were identified by analysis of variance (ANOVA) using appropriate general linear models, and multiple comparisons were made using the least significant differences (LSD) procedure (Statistix, Analytical Software, Tallahassee, FL). Differences were considered to be significant when $P \leq 0.05$. The numbers of replicates per treatment group and independent experiments associated with specific studies are provided in the figures or accompanying legends.

Results and Discussion

Effects of 4-AP on GC Viability and Progesterone Production

Treatment with 4-AP (2 mM) decreased progesterone accumulation in the media of serum-supplemented GC cultures at 24 h and 48 h post-treatment, in the presence and absence of FSH (Figure 1). The decreased progesterone concentrations in the media of drug-treated cultures did not reflect significant differences in the number of viable cells under these culture conditions, (Figure 2A). Densitometric analysis of data from 3 experiments similar to that shown in Figure 2B revealed no significant effect of 4-AP treatment on the expression of proliferating cell nuclear antigen (PCNA). These data provide additional evidence that 4-AP exerts neither an anti-proliferative nor a pro-apoptotic influence on serum-supplemented GC cultures, because PCNA expression is a sensitive marker of GC proliferation and apoptosis [18,26,27]. The lack of 4-AP effect on GC viability is also evident from the flow cytometric analysis; the percentages of cells staining positive for propidium iodide were 8 ± 2% and 7 ± 2% in the presence and absence of drug (n = 4 samples of 10,000 cells/each from 4 GC isolations).

Treatment with 4-AP (2 mM) also diminished progesterone accumulation on days 1 and 2 post-treatment in GC cultures maintained in the defined serum-free media in the presence and absence of FSH. However, in these serum-free cultures, 4-AP treatment was associated with not only decreased progesterone accumulation but also decreased numbers of viable cells (Table 1). The percentage of viable cells was unaffected by 4-AP (Control = 90 ± 4% vs. 4-AP = 88 ± 4%, n = 5; FSH = 86 ± 2%, FSH+4-AP = 86 ± 2%, n = 9), suggesting that 4-AP inhibited growth rather than promoted cell death.

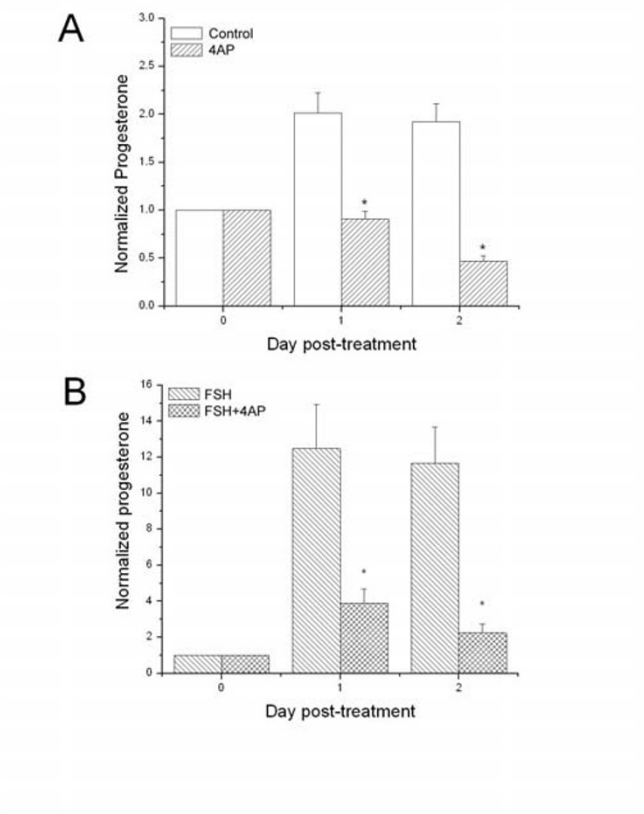

Figure 1
1 4-AP decreased progesterone output by serum-supplemented granulosa cell cultures. A) Progesterone accumulation by 24 h GC cultures maintained in the basic culture media containing 10% FBS (Control) in the absence or presence of 4-AP (2 mM). B) Progesterone accumulation by 24 h GC cultures maintained in the basic culture media in the absence or presence of added FSH (200 ng/mL) and/or 4-AP (2 mM). Data are normalized to day 0 progesterone concentrations, and represent the mean ± SEM of either 33 culture wells from 6 GC harvests (panel A) or 17 culture wells from 4 GC harvests (panel B). Asterisks indicate $P < 0.05$ compared with GC cultured under similar conditions in the absence of 4-AP.

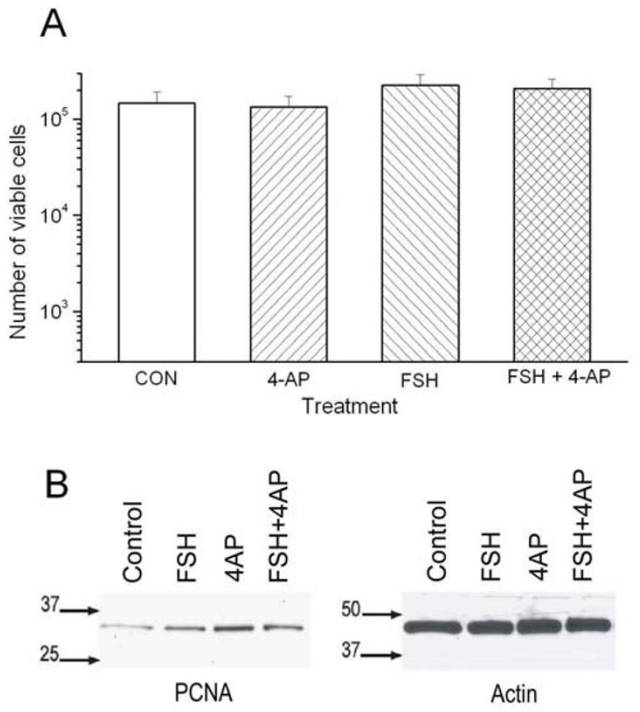

Figure 2
4-AP does not affect granulosa cell viability in serum-supplemented cultures. A) The numbers of viable cells (mean ± SEM) 24 h after treatment for GC cultures maintained in the absence (CON) of FSH with and without the addition of 2 mM 4-AP (10 culture wells from 5 different GC isolations), and in the presence of FSH with and without the addition of 2 mM 4-AP (8 culture wells from 4 GC isolations). B) Western blot analysis of proliferating cell nuclear antigen (PCNA, left) and actin (loading control, right) in GC lysates (40 μg protein/lane) from cultures described in panel A. Arrows indicate molecular mass (kDa). The results shown are representative of 3 independent experiments.

Overall, these data are consistent with previous reports that endocrine cells express voltage-sensitive ion channels that can contribute to the regulation of not only resting membrane potential and cell volume, but also cell proliferation and steroidogenesis [12,13,16,18,21,28–37]. The K^+ currents blocked by 4-AP in pig GC are conducted by ion channels formed by heteromeric complexes of pore-forming subunits from the KCNA (also called Kv1 or Shaker) family of proteins and accessory subunits from the KCNAB (also called Kvβ) family of proteins [4]. Similar 4-AP-sensitive K^+ currents play a key role in transduction of mitogenic signals in a variety of cell types, and 4-AP treatment has been associated with not only growth arrest but also apoptosis [2,3,8–10,14,38–42].

Our finding that 24 h exposure to 4-AP decreased the number of viable GC in serum-free but not serum-supplemented primary cultures is consistent with the reported effects of 4-AP and other K^+ channel antagonists on other cell types [8,43,44]. It has been shown that the concentrations of K^+ channel antagonists required to inhibit growth of human bladder tumor cells can be 70 times higher in the presence than the absence of serum [43]. 4-AP has

Table 1: 4-AP Effects on Granulosa Cells Cultured in Defined Serum-free Media

Variable	Treatment	Day 0	Day 1	Day 2
Media Progesterone Accumulation (ng/ml) (n = 12)	CONTROL	50.7 ± 6.9	314.9 ± 45.7	519.7 ± 87.7
	4-AP	40.2 ± 4.8	46.8 ± 3.9[a]	25.2 ± 4.3[a]
	FSH	43.0 ± 7.0	653.5 ± 86.1	623.2 ± 95.3
	FSH + 4-AP	52.9 ± 8.0	206.8 ± 55.9[b]	101.0 ± 26.8[b]
Number of Viable Cells (cells/well × 10^5) (n = 3)	CONTROL	2.7 ± 0.2	7.4 ± 1.9	6.3 ± 0.7
	4-AP	3.4 ± 1.3	2.2 ± 0.8[a]	2.2 ± 0.3[a]
	FSH	2.8 ± 0.5	5.5 ± 0.1	5.6 ± 0.0
	FSH + 4-AP	4.1 ± 1.1	3.8 ± 0.2[b]	3.0 ± 0.2[b]

[a] Signficantly different from control on same day post-treatment. [b] Signficantly different from FSH on same day post-treatment.

been shown to inhibit the proliferation of human myelobastic leukemia cells by preventing growth factor activation of mitogen activated protein kinase (MAPK) pathways [42]. This mechanism may be responsible for the anti-proliferative effect of 4-AP manifest in Table 1. It is likely that MAPK pathways are less robust in GC grown in defined serum-free vs. serum-supplemented media; however, validation of this hypothesis would require additional experiments beyond the scope of the present investigation. We concluded that the anti-proliferative effect of 4-AP may contribute to the decreased progesterone accumulation observed in serum-free but not serum-supplemented GC cultures, then focused additional efforts on understanding other mechanisms responsible for 4-AP inhibition of progesterone production.

The effects of 4-AP in serum-supplemented GC cultures are consistent with previous reports that antagonism of voltage-sensitive K^+ currents can modulate basal and gonadotropin-stimulated progesterone accumulation in the absence of any effect on cell growth or death. We have shown previously that antagonism of the slowly activating non-inactivating granulosa K^+ current (I_{Ks}) enhances basal progesterone production by depolarizing membrane potential and thus enhancing calcium influx via pimozide-sensitive Ca^{2+} channels [18]. In contrast, Kusaka [21] showed that antagonism of 4-AP sensitive slowly inactivating delayed rectifier K^+ currents suppressed both basal and LH-stimulated progesterone production. Data presented in Figures 1 and 2 confirm the inhibitory effects of 4-AP on basal progesterone production by cultured pig GC, and demonstrate for the first time a similar inhibitory effect of 4-AP on FSH-stimulated progesterone synthesis.

Effects of 4-AP on Pathways for Steroid Hormone Biosynthesis

The inhibitory action of 4-AP on progesterone production was investigated further using not only primary cultures, but also the spontaneously established granulosa cell line, PGC-2[19]. Although PGC-2 lack FSH receptors, these cells can serve as a useful *in vitro* model for GC function, because they produce cAMP in response to forskolin and synthesize progesterone and estradiol when supplied with appropriate substrates. We examined the effects of 4-AP on cAMP, progesterone and estradiol production by PCG-2 after confirming that these cells express 4-AP sensitive voltage-gated K^+ currents similar to those in primary cells (see additional file 1).

4-AP decreased production of cAMP (Figure 3A), and expression of StAR by PGC-2 (Figure 3B). 4-AP had no significant effect on progesterone production by PCG-2 cultured in the presence of pregnenolone, androstenedione or 5α-DHT (Figure 4A). In contrast, 4-AP significantly reduced estradiol production by PGC-2 cultures provided with androstenedione (Figure 4B).

To evaluate the relevance of the experimental results obtained using PGC-2, we examined the effect of 4-AP on the progesterone output of primary GC cultures supplemented with either the membrane permeable cAMP analog, 8-(4-chlorophenylthio) adenosine-3',5'-cyclic monophosphorothioate (8-CPT-cAMP, 1 mM) or estradiol 17β (500 ng/ml). The inhibitory effect of 4-AP on progesterone accumulation was partially but not completely overcome by the addition of 8-CPT-cAMP (Table 2). 4-AP had no inhibitory effects on either basal or FSH-stimulated progesterone production by GC cultures supplemented with estradiol 17β (Figure 5). Thereby, the combined results of experiments performed using PGC-2 and primary GC cultures suggest that 4-AP-inhibition of GC progesterone

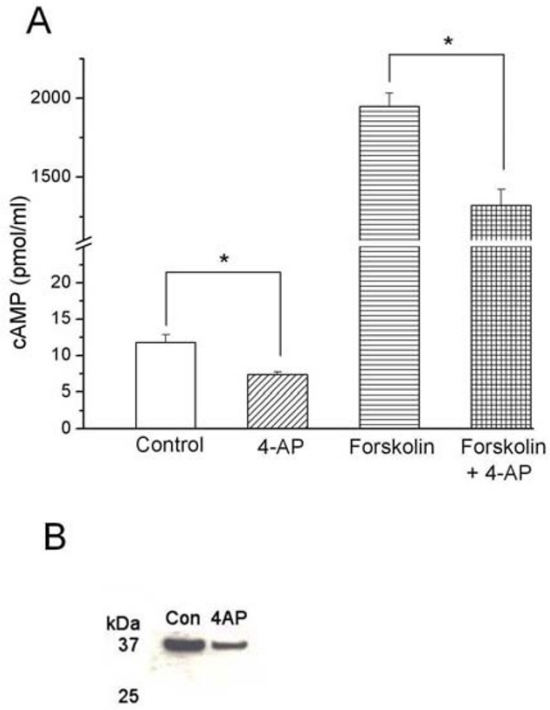

Figure 3
4-AP decreases cAMP and StAR in PGC-2 cultures. A) Accumulation of cAMP in PGC-2 (n = 4 culture wells). PGC-2 were cultured in serum-free media (McCoy's modified 5A) in the presence of IBMX (10 μM) without (Control) or with the addition of 4-AP (2 mM) and forskolin (5 μM) for 2 h, then pelleted and lysed in HCl (0.1 N) prior to analysis of cAMP levels. B) Western blot analysis of StAR in mitochondrial extracts from PGC-2 cells (10 ug protein/lane) cultured in serum-free media for 2 h in the absence (Con) or presence of 4-AP. The results shown are representative of 3 independent experiments. Astericks indicates $P < 0.05$ compared with PGC-2 cultures under similar conditions in the absence of 4-AP.

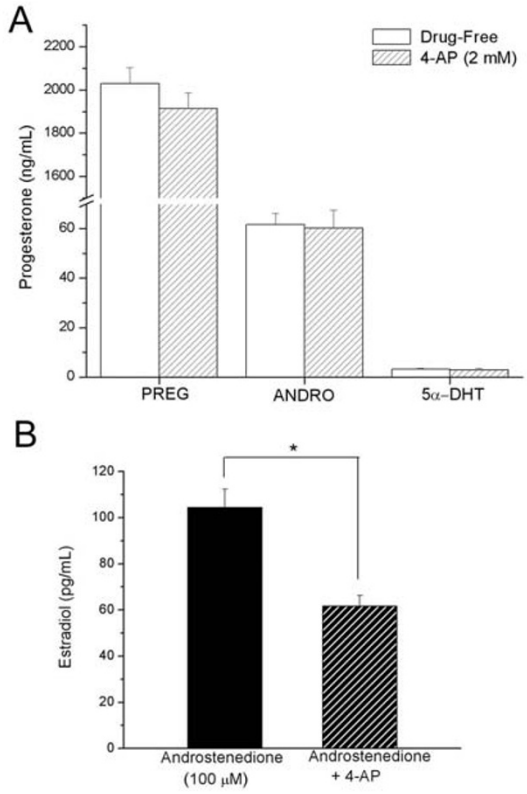

Figure 4
4-AP decreases estradiol production by PGC-2 cultures. A) Accumulation of progesterone in media of PGC-2 (n = 4 culture wells) cultured with pregnenolone (1 μg/ml), androstenedione (100 μM), or 5α-dihydrotestosterone (5α-DHT, 100 μM). B) Accumulation of estradiol in media of PGC-2 (n = 4 culture wells) cultured with androstenedione (100 μM). In all panels, asterisk indicates $P < 0.05$ compared with PGC-2 cultured under similar conditions in the absence of 4-AP.

production may be linked to decreased expression of StAR, decreased generation of cAMP, and decreased output of estradiol.

StAR plays a key role in the initial steps of steroidogenesis, because it is required for transport of cholesterol to the inner mitochondrial membrane where CYP11A1 resides and the first reaction in progesterone synthesis occurs [45]. cAMP is a well known second messenger in FSH- and LH-stimulated progesterone synthesis, and a key effect of the cAMP-dependent protein kinase in GC is transcriptional control of StAR [46,47]. Estradiol is recognized as a biological amplifier of basal, FSH- and cAMP-stimulated progesterone production in pig GC, with significant synergistic effects distal to the generation of cAMP at one or more steps in cholesterol transport and metabolism [48]. Our experimental results are thus consistent with 4-AP inhibition of GC progesterone output via drug effects on multiple interacting sites in the steroidogenic pathway. Moreover, despite multiple sites of action, the drug's actions cannot be dismissed as non-specific, because 4-AP had no effects on either 3β-hydroxysteroid dehydrogenase (3β-HSD)-mediated conversion of pregnenolone to progesterone, or androgen (androstenedione, 5αDHT)-stimulated progesterone synthesis.

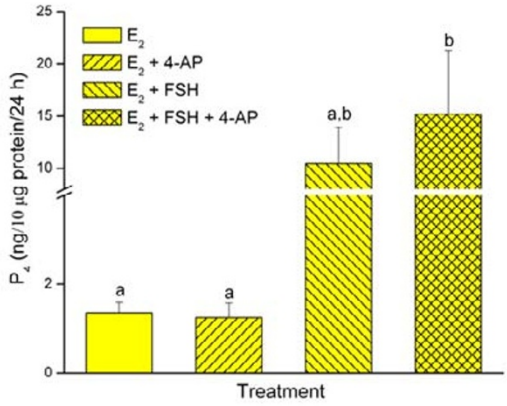

Figure 5
Estradiol prevents 4-AP inhibition of progesterone production by granulosa cell cultures. Progesterone (P_4) accumulation by 24 h GC cultures maintained in the basic culture media supplemented with 500 ng/mL estradiol 17β (E_2), in the absence or presence of FSH (200 ng/mL), with or without the addition of 4-AP (2 mM). Data were obtained from 9 (E_2+FSH) or 10 (E_2, E_2+4-AP, E_2+FSH+4-AP) culture wells from two GC isolations. Different superscripts indicate significant difference ($P < 0.05$).

Estradiol has been reported to affect K^+ channel expression and gating in smooth muscle [49–53]. The data presented here do not address directly the potential for estradiol modulation of voltage-gated K^+ channel expression or activity in GC. However, such a mechanism seems unlikely to account for the ability of estradiol to oppose the inhibitory effects of 4-AP on GC progesterone production. The K^+ channel subunits and currents modulated by estradiol in vascular and uterine smooth muscle differ from the 4-AP sensitive KCNA family channels that contribute to the 4-AP sensitive current I_{Kur} in pig GC [4,49,50,52,53]. Moreover, in the single study where estradiol was shown to influence the gating of a uterine delayed rectifier current with electrophysiological characteristics resembling those of GC I_{Kur}, the observed effect of the hormone was inhibitory [51], and thereby similar rather than opposite to the effect of 4-AP.

Additional experiments were performed in an attempt to identify signaling pathways that transduce specific inhibitory effects of 4-AP. These focused on 4-AP modulation of membrane potential, intracellular ion concentrations, and cell size, as described below.

Effects of 4-AP on Membrane Potential, Intracellular Ions, Cell Size

Hormonal signal transduction and steroidogenesis can be affected by changes in intracellular and extracellular concentrations of inorganic ions, cellular resting membrane potential, and cell size [29,30,35,54–59]. Blockade of 4-AP sensitive K^+ channels in non-nerve, non-muscle cells can lead to changes in transmembrane ion gradients, membrane potential and cell volume [42,60]. On this basis, we determined the effects of 24 h exposure to 4-AP on GC size, membrane potential and intracellular potassium ($[K^+]_{in}$).

Samples (10,000 cells) of GC from 24 hour cultures associated with 4 GC isolations were analyzed by flow cytometry to determine if 4-AP treatment influenced GC size. Light scattering properties did not differ significantly with GC isolate. However, the average forward scatter (median, coefficient of variation) of GC cultured for 24 hours in the presence of 4-AP (768, 15%) was significantly greater than that of GC maintained similarly in the absence of drug (694, 15%). Representative dot plots are shown in Figure 6. Resting membrane potential (Figure 7A) and $[K^+]_{in}$ (Figure 7B) were also found to be increased in GC exposed to 4-AP, based on $DiBAC_4(3)$ and PBFI fluorescence, respectively.

The observed drug-induced increases in cell size and $[K^+]_{in}$ suggest that 4-AP sensitive K^+ channels represent a significant K^+ efflux pathway involved in maintenance of cell volume in GC. Interestingly, the increased $[K^+]_{in}$ may contribute directly to the inhibitory effects of 4-AP on production of cAMP and progesterone. Loss of K^+ from the intracellular compartment of rat GC has been associated with opposite effects, namely enhanced synthesis of both cAMP and progesterone [61].

The observed increase (hyperpolarization) of GC resting membrane potential cannot be explained easily by any direct effect of the drug. In fact, 4-AP inhibition of K^+ efflux would be expected to depolarize rather than hyperpolarize membrane potential. Data obtained previously in our laboratory suggest that inhibition of granulosa I_{Kur} should be associated with a +10 to +20 mV depolarization of cell resting membrane potential; block of I_{Ks} alone decreased resting potential by +10 mV while block of both I_{Ks} and I_{Kur} decreased resting potential by +20 to +30 mV [18]. Most previous investigations of 4-AP effects on the resting membrane potentials of non-nerve, non-muscle cells have similarly demonstrated drug-induced depolarization [42,60], although there is a single report showing 4-AP-induced hyperpolarization of chondrocyte membrane potential [62].

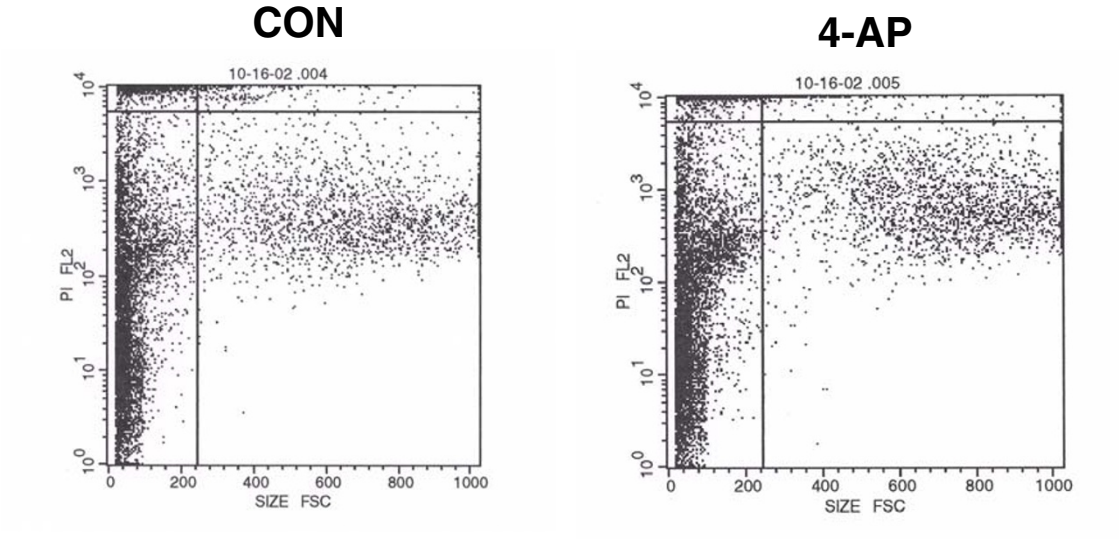

Figure 6
4-AP increases granulosa cell size. Dot plots of forward scatter (SIZE FSC) vs. propidium iodide fluorescence (PI FL-2) for granulosa cells cultured in either the absence (CON) or presence of 4-AP (2 mM). Each dot represents one cell. Dead cells stained with propidium iodide appear in two upper quadrants. Cellular debris and shrunken apoptotic cells are displayed in the lower left quadrant. Viable granulosa cells are represented in the lower right quadrant. Median forward scatter was 637 and 735 for viable CON and 4-AP, respectively.

Table 2: 4-AP Effects on cAMP-stimulated Progesterone Accumulation (ng/ml)

Treatment	Day 0	Day 1
CONTROL (n = 4)	10.3 ± 3.6[a]	60.1 ± 25.2[b]
8-CPT-cAMP (n = 4)	7.1 ± 0.5[a]	792.4 ± 83.6[c]
8-CPT-cAMP + 4-AP (n = 7)	11.4 ± 2.1[a]	380.7 ± 93.5[d]

Groups with different superscripts are significantly different.

We hypothesized that hyperpolarization of 4-AP-treated GC could reflect anion influx associated with activation of a Cl^- entry pathway sensitive to changes in cell volume as well as membrane-potential. To test this hypothesis, the effects of 4-AP on membrane potential were examined using GC incubated in either media modified to contain the chloride channel antagonist DIDS, or a physiological buffer solution modified to contain a low (8 mM) concentration of chloride. In contrast to the results obtained in the standard media (Figure 7A), 4-AP did not induce significant hyperpolarization of GC under these conditions (Figure 8A,8B). Exposure to DIDS alone significantly decreased (depolarized) GC resting membrane potential (Figure 8B), suggesting that an outward chloride current (chloride influx) contributes to the normal resting potential of pig GC. Membrane potential was further depolarized in GC exposed to 4-AP in the presence of DIDS

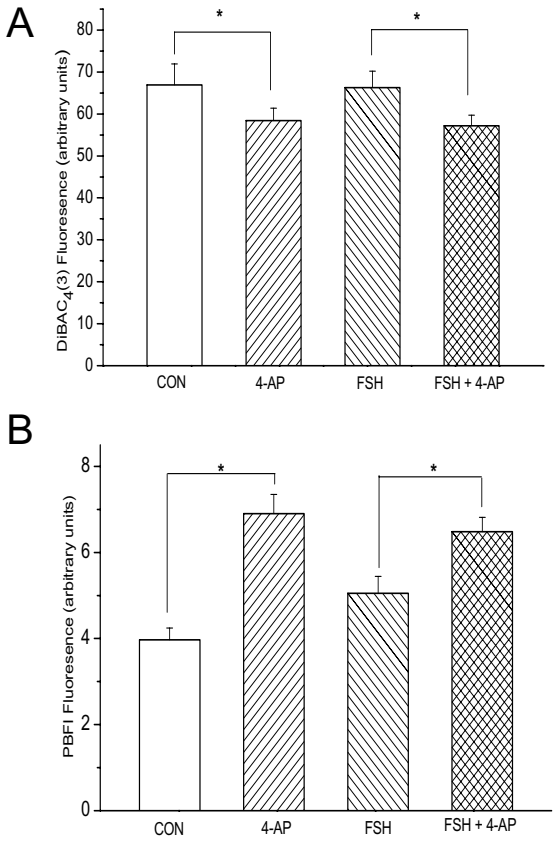

Figure 7
4-AP hyperpolarizes granulosa cell resting potential and increases intracellular potassium. A) Granulosa cells (GC) were cultured in the basic media, alone (Con), or with 4AP (2 mM), FSH (200 ng/mL) or FSH+4AP. After 24 hours, resting membrane potentials were compared using $DiBAC_4(3)$ fluorescence. Decreased $DiBAC_4(3)$ fluorescence indicates hyperpolarization of resting membrane potential. Data were obtained from 16 (Con, 4-AP) or 17 (FSH, FSH+4AP) culture wells from 4 GC isolations. B) GC were cultured in the basic media, alone, or with 4AP, FSH or FSH+4AP. After 24 hours, intracellular K^+ concentrations were compared by PBFI fluorescence. Increased PBFI fluorescence indicates increased intracellular potassium concentration. Data were obtained from 5 (FSH, FSH+4-AP) or 7 (Con, 4-AP) culture wells from 3 GC isolations. In both panels, asterisk indicates $P < 0.05$ compared with GC cultured under similar conditions in the absence of 4-AP.

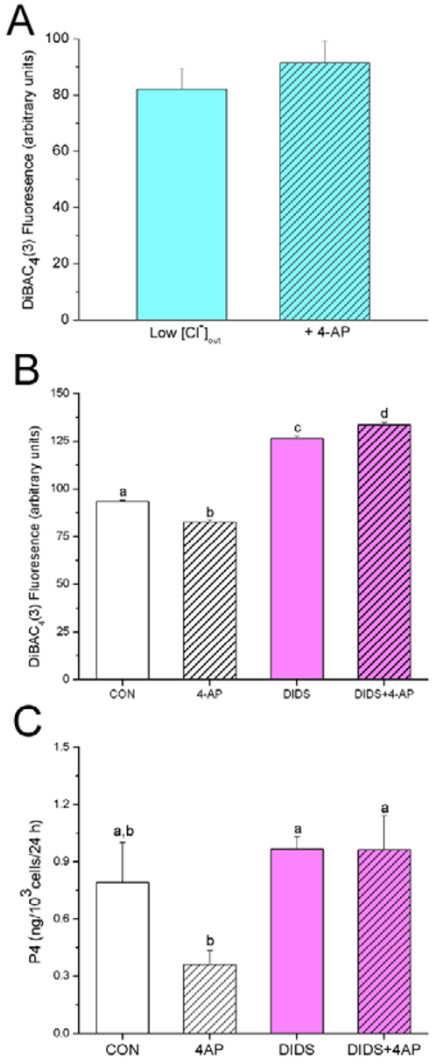

Figure 8
Chloride influx mediates the effect of 4-AP on granulosa cell membrane potential and progesterone production. A) Effect of 4-AP (2 mM) on membrane potential of granulosa cells (GC) bathed in Tyrode solution modified to contain a low concentration (8 mM) of chloride (Low $[Cl^-]_{out}$). Data represent $DiBAC_4(3)$ fluorescence of untreated GC (n = 8) and GC exposed to 4-AP for 0.5 h (n = 7). B) Effect of DIDS (100 μM) and 4-AP (2 mM) on membrane potential ($DiBAC_4(3)$ fluorescence) of GC (n = 6) cultured for 24 h in the presence or absence of drugs. Decreased $DiBAC_4(3)$ fluorescence indicates hyperpolarization of resting membrane potential, while increased $DiBAC_4(3)$ fluorescence indicates depolarization of resting membrane potential. C) Progesterone (P_4) accumulation by 24 h GC cultures maintained in the basic culture media (CON) alone or with 4-AP (2 mM), DIDS (100 μM) or DIDS+4AP. Data were obtained from 4 (CON, 4-AP) or 6 (DIDS, DIDS+4-AP) culture wells from a single GC isolation. Different superscripts indicate significant difference ($P < 0.05$).

(Figure 8B), consistent with the hypothesis that 4-AP-induced hyperpolarization depends on chloride influx.

Interestingly, concomitant exposure to DIDS prevented 4-AP from diminishing the accumulation of progesterone in GC culture media (Figure 8C), suggesting that chloride influx, membrane hyperpolarization or both might mediate this effect of 4-AP. Chloride channels or chloride ions are known to influence cAMP-stimulated steroidogenesis by adrenal cells [29], human chorionic gonadotropin (hCG)-stimulated steroidogenesis by amphibian follicle-enclosed oocytes [56], as well as LH-stimulated steroidogenesis in rat Leydig cells [29], MA-10 cells [59], and chicken GC [35]. In mammalian cells, removal of extracellular chloride stimulates basal and protein kinase A (PKA)-dependent steroid hormone production by increasing the expression of StAR and potentiating the effects of submaximal concentrations of cAMP [29,59]. The proposed mechanism of action involves chloride efflux, depolarization of the mitochondrial and plasma membranes, and alterations to protein synthesis and cholesterol transport [29,59].

The data shown here in Figures 7 and 8 suggest strongly that chloride ions can influence progesterone production in pig GC by similar mechanisms. In our experiments, the chloride influx and membrane hyperpolarization associated with 4-AP treatment not only decreased expression of StAR, but also inhibited cAMP production and steroidogenesis. The observed effects of Cl^- channel antagonists differ significantly between the present study where DIDS prevented 4-AP from decreasing progesterone synthesis and previous investigations where DIDS and other Cl^- channel antagonists prevented LH from enhancing progesterone production [29,35,56]. However, this discrepancy is expected, because DIDS antagonizes a chloride influx pathway in pig GC, and chloride efflux pathways in Leydig cells.

In summary, the data demonstrate that 4-AP inhibits basal and FSH-stimulated progesterone production by pig GC via a complex mechanism involving drug action at multiple interacting steps in the steroidogenic pathway. Drug block of the voltage-gated potassium channels that conduct the ultra-rapid delayed rectifier current I_{Kur} is associated with increased intracellular potassium concentration, increased cell volume, activation of chloride influx and hyperpolarization of resting membrane potential. These changes in the GC internal milieu appear to modulate the inhibitory effects of 4-AP on expression of StAR and production of steroid hormones.

The physiological implications of the data are significant, as they suggest novel roles for granulosa cell ion channels. We demonstrate for the first time not only that delayed rectifier K^+ channels play a significant role in GC volume regulation, but also that voltage and/or volume-sensitive Cl^- channels modulate GC membrane potential and progesterone production. Additional experiments are required to link these data obtained *in vitro* to functionally significant changes in GC volume and chloride flux during normal follicular development.

Conclusions

In conclusion, the present studies indicate that the K^+ channel antagonist 4-AP inhibits basal and FSH-stimulated progesterone production by pig GC. Exposure to 4-AP is associated with decreased production of cAMP, decreased expression of StAR, and decreased synthesis of estradiol. These inhibitory actions of 4-AP on adenylate cyclase activity, StAR expression and steroidogenesis may reflect drug-induced changes in the intracellular concentrations of K^+ and Cl^- and the GC resting membrane potential. Further studies are required to elucidate fully the mechanisms involved.

Additional investigation is warranted. 4-AP is reported to have therapeutic potential for the relief of symptoms associated with demyelinating diseases such as spinal cord injury and multiple sclerosis [63–68]. The drug is currently being studied in large scale human trials [68]. Drug-induced inhibition of steroid hormone production could have significant effects in these patients. For example, 4-AP could exacerbate abnormal steroid hormone profiles associated with spinal cord injury and multiple sclerosis, and thereby contribute to dysmenorrhea and infertility [69,70].

List of Abbreviations

4-aminopyridine (4-AP); analysis of variance (ANOVA); 8-(4-chlorophenylthio)adenosine-3',5'-cyclic monophosphorothioate (8-CPT-cAMP); 4',4' diisothicyanato-stilbene-2-2'-disulfonic acid (DIDS); bis-(1,3-dibutylbarbituric acid)trimethine oxonol ($DiBAC_4(3)$); 5α-dihydrotestosterone (5α-DHT); Dulbecco's modified eagle's medium (DMEM); enhanced chemiluminescence (ECL); ethylenediaminetetraacetic acid (EDTA); fetal bovine serum (FBS); follicle stimulating hormone (FSH); granulosa cells (GC); human chorionic gonadotropin (hCG); 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES); 3β-hydroxysteroid dehydrogenase (3β-HSD); 3-isobutyl-1-methylxanthine (IBMX); least significant difference (LSD); luteinizing hormone (LH); mitogen activated protein kinase (MAPK); 1,3-Benzenedicarboxylic acid, 4,4'-[1,4,10,13-tetraoxa-7,16-diazacyclooctadecane-7,16-diylbis(5-methoxy-6,2-benzofurandiyl)]bis-, tetrakis [(acetyloxy)methyl] ester (PBFI-AM); phosphate-buffered saline (PBS); proliferating cell nuclear antigen (PCNA); protein kinase A (PKA); sodium dodecyl sulphate-polyacrylamide gel electrophoresis

(SDS-PAGE); steroidogenic acute regulatory protein (StAR); tris-buffered saline (TBS).

Authors' contributions

YL participated in experimental design, performed all experimental techniques, and played a primary role in data analysis and interpretation. SG provided general technical support and participated in cell isolation and culture, immunoblotting, fluorescence measurements and flow cytometry. FvS performed patch clamp experiments and analyses, and assisted with preparation of the figures and manuscript. DEM contributed to data presented in Figure 1 and Table 1. BMM contributed to data presented in Figures 1 and 2. LCF conceived of the study, coordinated design and performance of experiments, and drafted the manuscript.

Additional material

Additional File 1

*4-AP-sensitive delayed rectifier K^+ current in PGC-2. Whole-cell patch clamp currents recorded from a PGC-2 cell, in response to a series of six depolarizing test pulses of 1 s duration (step +20 mV) from a holding potential of -50 mV, before (left) and after (middle) application of 2 mM 4-aminopyridine (4-AP). The right panel shows the 4-AP sensitive current obtained by subtracting the current traces recorded in the presence (middle) of the drug from those recorded in the absence (left) of the drug. Recording conditions are identical to those used previous by our laboratory to record voltage-dependent K^+ currents from pig granulosa cells (*Mol Pharmacol *2002 61:201-13). The data demonstrate that PGC-2 cells express K^+ currents similar to the granulosa cell ultra-rapid delayed rectifier current (I_{Kur}) in terms of the voltage-dependence and kinetics of activation and deactivation, as well as the sensitivity to 4-AP. Similar currents were recorded in 7 of 7 cells assayed using patch-clamp techniques.*

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