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Mechanisms of Intracellular Calcium Homeostasis in Developing and Mature Bovine Corpora Lutea[1]

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ABSTRACT

Although calcium (Ca^{2+}) is accepted as an intracellular mediator of prostaglandin F2 alpha (PGF2alpha) actions on luteal cells, studies defining mechanisms of Ca^{2+} homeostasis in bovine corpora lutea (CL) are lacking. The increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) induced by PGF2alpha in steroidogenic cells from mature CL is greater than in those isolated from developing CL. Our hypothesis is that differences in signal transduction associated with developing and mature CL contribute to the increased efficacy of PGF2alpha to induce a Ca^{2+} signal capable of inducing regression in mature CL. To test this hypothesis, major genes participating in Ca^{2+} homeostasis in the bovine CL were identified, and expression of mRNA, protein, or activity, in the case of phospholipase Cbeta (PLCbeta), in developing and mature bovine CL was compared. In addition, we examined the contribution of external and internal Ca^{2+} to the PGF2alpha stimulated rise in $[Ca^{2+}]_i$ in LLCs isolated from developing and mature bovine CL. Three differences were identified in mechanisms of calcium homeostasis between developing and mature CL, which could account for the lesser increase in $[Ca^{2+}]_i$ in response to PGF2alpha in developing than in mature CL. First, there were lower concentrations of inositol 1,4,5-trisphosphate (IP3) after similar PGF2alpha challenge, indicating reduced phospholipase C beta (PLCbeta) activity, in developing than mature CL. Second, there was an increased expression of sorcin (SRI) in developing than in mature CL. This cytoplasmic Ca^{2+} binding protein modulates the endoplasmic reticulum (ER) Ca^{2+} release channel, ryanodine receptor (RyR), to be in the closed configuration. Third, there was greater expression of ATP2A2 or SERCA, which causes calcium reuptake into the ER, in developing than in mature CL. Developmental differences in expression detected in whole CL were confirmed by Western blots using protein samples from steroidogenic cells isolated from developing and mature CL. Localization of these genes in steroidogenic luteal cells was confirmed by immunohistochemistry. Therefore, it is concluded that the cellular mechanisms that allow PGF2alpha to induce a calcium signal of greater magnitude in mature than in developing CL involve 1) greater PLCbeta activity with enhanced generation of IP3, 2) an enhanced Ca^{2+} release from the ER via unrestrained RYR2 due to a decrease in SRI expression, and 3) a reduction in calcium reuptake to the ER due to lower expression of ATP2A2. Accordingly, the increase in $[Ca^{2+}]_i$ induced by PGF2alpha in mature large steroidogenic cells had less dependency from extracellular calcium than in those isolated from immature CL.

calcium homeostasis, corpus luteum, luteal physiology, luteal sensitivity to PGF2α, ovary

INTRODUCTION

Exogenous PGF2α is less effective in inducing regression of corpora lutea (CL) and subsequent ovulation in cattle if administered when CL are developing (Days 1–5) rather than mature [1–7]. Fully understanding the cellular mechanisms responsible for this difference in response to exogenous PGF2α is important for successful development of more effective protocols for synchronization of ovulation. The importance of this physiological difference is reflected in the intensity with which this area has been investigated [8–12]. In spite of this research effort, there is a fundamental gap in understanding the cellular basis responsible for the developmental difference in luteal response to exogenous PGF2α. In the cow, differences in expression of functional PGF2α receptor (FP or PTGFR) cannot explain the differential response of developing and mature CL to exogenous PGF2α [13], which was confirmed in this study.

PGF2α exerts effects on target luteal cells by binding to cognate Gq-coupled FP receptors and activating phospholipase C (PLC) [14–16]. On PLC activation, inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG) are generated from plasma membrane phosphatidylinositol 4,5-bisphosphate (PIP_2) [17]. IP3 diffuses through the cytoplasm and binds to IP3 receptors (ITPRs) on the endoplasmic reticulum (ER), causing them to open and release calcium ions (Ca^{2+}) into the cytoplasm [17]. The PGF2α-stimulated rise in cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_i$) was demonstrated to occur in large (LLCs) and small (SLCs) bovine luteal cells [17–19], ovine LLCs [20, 21], human granulosa-luteal cells [22], and primate CL [23]. Therefore, DAG and Ca^{2+} activate conventional protein kinase C (PKC) and are thought to be intracellular mediators of PGF2α actions in luteal cells [24].

Choudhary et al. [19] suggested that lower efficacy of PGF2α in developing CL was likely related to differences in components of signal transduction associated with FP receptors at these two developmental stages. This suggestion was based on the observation that the magnitude of the increase in $[Ca^{2+}]_i$ stimulated by PGF2α was greater in bovine steroidogenic cells

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isolated from mature compared to those of developing CL [19]. The importance of this rise in $[Ca^{2+}]_i$ as a mediator of PGF2α actions in bovine luteal cells was demonstrated in vitro by abolishing the inhibitory effect of PGF2α on luteinizing hormone (LH)-stimulated progesterone production under conditions that prevented an elevation of $[Ca^{2+}]_i$ [25]. Additionally, increasing $[Ca^{2+}]_i$ by pharmacological means in developing bovine luteal cells reduced the stimulatory effect of LH on progesterone secretion, an effect that cannot be induced by PGF2α at this developmental stage [25]. Several authors have stressed the importance of this increase in $[Ca^{2+}]_i$ in inhibiting progesterone production by bovine LLCs, [26], mature ovine LLCs, [21] and mature primate CL [23, 27].

As expected, due to the critical regulatory role of Ca^{2+} in cell physiology, $[Ca^{2+}]_i$ is tightly regulated by the combined actions of Ca^{2+} channels, Ca^{2+} metabolic pumps, and numerous Ca^{2+} binding proteins [28] (Fig. 1). A particular cytoplasmic calcium-binding protein of interest is sorcin, which has been shown to associate with ryanodine receptors and with L-type calcium channels, thereby modulating Ca^{2+} release and influx. Calcium ions can enter the cytoplasm primarily from two sources: the extracellular milieu via plasma membrane channels and internal stores, such as the ER, via calcium-release channels, such as inositol 1,4,5-trisphosphate receptors (ITPRs) and ryanodine receptors (RYRs). In mouse luteinized granulosal cells, calcium release from the ER into the cytoplasm was demonstrated to occur through ITPRs and through RYRs via a calcium-induced calcium release (CICR) mechanism [29]. Both ITPRs and RYRs are present on the ER of mouse Leydig cells, but RYRs were determined to be the principal channels that increased $[Ca^{2+}]_i$ [30]. Furthermore, in ovine LLCs, PGF2α was shown to stimulate Ca^{2+} release from ER stores [31]. The contribution of the extracellular milieu in increasing $[Ca^{2+}]_i$ is mediated by members of the voltage-gated calcium channel (VGCC) family: L, N and T types [28, 32]. L- and T-type VGCCs were found in several types of steroidogenic cells, such as bovine adrenal glomerulosal cells, [33], mouse Leydig cells [34], and human granulosal cells [35].

The mechanisms of calcium homeostasis in bovine CL are incompletely known. Specifically, the identities of the genes involved in calcium homeostasis have not been determined. More important, it is unknown if there are changes in gene/protein expression or enzyme activity in one or several of these genes involved in calcium homeostasis, which could contribute to the developmental difference observed in the PGF2α-stimulated rise in $[Ca^{2+}]_i$. Therefore, this study tested the hypothesis that differences in luteal components involved in calcium homeostasis contribute, at least in part, to the ability of PGF2α to induce a rise in $[Ca^{2+}]_i$ in mature bovine CL. To test this hypothesis, we first identified major genes participating in calcium homeostasis in bovine CL (Table 1) and compared expression of mRNA, protein, or activity, in the case of phospholipase Cβ (PLCβ), in developing and mature bovine CL. Observations made in whole CL were extended and confirmed by using protein samples from steroidogenic cells isolated from developing and mature CL. Furthermore, localization of these genes in steroidogenic luteal cells was confirmed by immunohistochemistry. Finally, the contributions of external and internal calcium to the PGF2α stimulated rise in $[Ca^{2+}]_i$ were examined in LLCs isolated from developing and mature bovine CL.

MATERIALS AND METHODS

Animal Handling and Tissue Collection

Nonlactating beef cows were observed for estrus twice a day with the day of behavioral estrus designated as Day 0. Ovaries or CL were collected through supravaginal incision under epidural anesthesia (6–9 ml 2% lidocaine hydrochloride; Butler Company, Columbus, OH) from cows weighing 450–700 kg on Day 4 (developing CL) and Day 10 (mature CL) as described previously [19]. Tissues were transported to the laboratory in ice-cold saline for isolation cells, RNA, or protein. CL used for RNA isolation were different from those used in protein isolation. Day 4 and Day 10 CL processed to obtain enriched luteal steroidogenic cells were different from those described above. The protocol for tissue collection was reviewed and approved by the Animal Care and Use Committee of West Virginia University (ACUC no. 09–0608).

Isolation of Total RNA and cDNA Synthesis

Tissues from Day 4 (n = 4) and Day 10 (n = 4) bovine CL were homogenized individually and total RNA was isolated using Tri-reagent according to the manufacturer's instructions (Molecular Research Center) and as previously described [36]. Briefly, frozen CL tissue immersed in liquid nitrogen was pulverized mechanically using an RNase-free mortar and pestle. The pulverized tissue was homogenized in Tri-reagent using an RNase-free glass homogenizer. RNA samples were solubilized in RNase-free water. Total RNA concentration was determined via the NanoDrop spectrophotometer (ND-1000; NanoDrop Technologies). All RNA samples were assessed by 1% agarose gel electrophoresis and deemed of high quality by containing two distinct bands representing 28s rRNA and the 18s rRNA. DNA contamination was assessed by running RT control. This mock reverse transcription contained all the RT-PCR reagents, except the reverse transcriptase. This control allowed us to determine that the PCR amplification due to genomic DNA contamination was negligible compared to the one due to the cDNA sequence. One microgram of total RNA from luteal samples was used for cDNA synthesis reactions using Superscript II reverse transcriptase, and oligo-dT (18) (Invitrogen Life Technologies, Grand Island, NY) was used for both semiquantitative real-time PCR and semiquantitative RT-PCR.

Real-Time PCR

Messenger RNA expression was analyzed for gene transcripts via real-time PCR. Scientific symbols, gene names, forward and reverse primer sequences, and accession numbers for each gene are listed in Table 1. Efficiencies for each primer set were calculated using a 10-fold serial dilution of the pooled stock cDNA (Days 4 and 10). This analysis produced a calibration curve and slope used for the efficiency-calibrated model [37]. The real-time PCR reaction (25 μl) included 2× SYBR Green Supermix, 0.5 μM primer concentration, and 2 μl

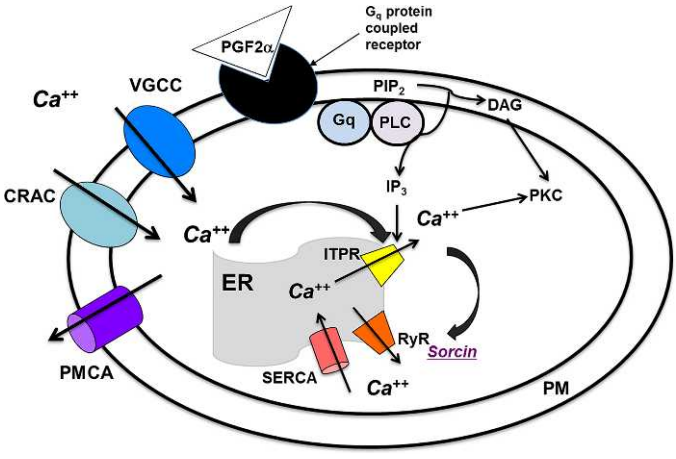


FIG. 1. Mechanisms of calcium homeostasis in a generic cell. Calcium ions (Ca^{2+}), plasma membrane (PM), endoplasmic reticulum (ER), voltage-gated calcium channels (VGCC), Orai/STIM or Ca^{2+}-release activated Ca^{2+} channels (CRAC), plasma membrane Ca^{2+}-ATPase pumps (PMCA), phospholipase C (PLC), phosphatidylinositol 4,5-bisphosphate (PIP_2), inositol 1,4,5-trisphosphate (IP3), diacylglycerol (DAG), IP3 receptors (ITPR), ryanodine receptors (RyR), conventional protein kinase C (PKC), sarco(endo)plasmic reticulum Ca^{2+}ATPase pumps (SERCA).

TABLE 1. *Bos taurus* gene-specific primers and their sequences for real-time PCR.

Gene symbol	Gene name	Forward primer	Reverse primer	Accession no.
Components of the endoplasmic reticulum				
ATP2A2	Calcium transporting, cardiac muscle, slow twitch 2 (SERCA2)	5′AAGAAGACAACCCGGACTTT	5′CAGGCAAGGAGATTTTCAGC	NM_001191430.1
ITPR1	Inositol 1,4,5-trisphosphate receptor, type 1	5′CAATGTCAACCCACAGCAAC	5′CGAGCTTTTTAACTGCAGCAAC	NM_174841.2
ITPR2	Inositol 1,4,5-trisphosphate receptor, type 2	5′CAAATCCAGCCCAGAAAGC	5′AATTCAGCCTGCATCATCG	NM_174369.2
ITPR3	Inositol 1,4,5-trisphosphate receptor, type 3	5′GAGCTGCCACATTTGTGAAC	5′TGATCACCCCAAAGATGAGG	NM_174370.2
RYR1	Ryanodine receptor, type 1	5′GGGAAGAGGAGGAACCAGAG	5′AGGAGGGGGAGATGGTGTAG	NM_001206777.1
RYR2	Ryanodine receptor, type 2	5′CAATACAGGCTTGTGGCTGA	5′GGTGGGTGGAAGTAGCCAAT	XM_002698813.1
RYR3	Ryanodine receptor, type 3	5′GCCCCTGTATGGACAGTGAT	5′CTCGCTCTTTGGGATGTAGC	XM_590220.4
SRI	Sorcin	5′CAAGATGCAGTACGGAGGG	5′CCTCCAGCAATGCCCGACT	NM_001075350.2
Components of the plasma membrane				
ATP2B1	ATPase, calcium transporting, plasma membrane 1	5′CCTGCTGCCGGGCGAGATATGT	5′CCCGCAGCTCTGCGAGTGTA	NM_174696.2
ATP2B2	ATPase, calcium transporting, plasma membrane 2	5′ACCAGGTGCGCAAGTCGGTG	5′CCGTCCTGCTGTTTGGCTTTCTTG	NM_001191245.1
ATP2B3	ATPase, calcium transporting, plasma membrane 3	5′GTGGGCTTCGCCATGGGCAT	5′TGATGCAGGCGCCCGTGAAG	NM_001191164.1
ATP2B4	ATPase, calcium transporting, plasma membrane 4	5′GCTGATGGAGCTGCGCTCAAGT	5′GTCCTCTGGGCTAGTGACGGC	NM_001172594.1
CACNA1C	Voltage-gated calcium channel alpha 1c subunit (L type)	5′CAGCACCTTGGAGGCCCAGG	5′CAAGCTGGGCTCGCTGCTGT	XM_002704472.1
CACNA1B	Calcium channel, voltage dependent, N type, alpha 1B subunit	5′CACTGCGGGCTGTGCGTGTA	5′CGCACGGGAAGTCACCCACC	NM_174632.2
CACNA1H	Calcium channel, voltage dependent, T type, alpha 1H subunit	5′GAGGGGGAACTTGGCGGTGC	5′TGCCGAGCATGGGCAGTGTG	XM_002697974.1
ORAI1	ORA1 calcium release-activated calcium modulator 1	5′GAGTTACTCCGAGGTGATGA	5′GACCGAGTTGAGATTGTGC	NM_001099002.1
ORAI2	ORA2 calcium release-activated calcium modulator 2	5′CATGGATTACCGGGACTG	5′CACGGGGAGGAACTTGAT	NM_001191348.1
ORAI3	ORA3 calcium release-activated calcium modulator 3	5′TTGCTGAAGTTGTCCTGG	5′TCCTCTAGTTCCTGCTTGTAG	NM_001193202.1
PLCB1	Phospholipase C beta-1	5′GGGTTCACCATGACCACAA	5′GCCACTTCCTTCTGACGACT	NM_174817.1
PLCD3	Phospholipase C delta-3	5′TCCTGAAGAACGGGAAGTTG	5′CTCCCTCTCCCTCCTTGATT	NM_001193028.1
PLCG1	Phospholipase C gamma-1	5′CACCGTCATGACTTTGTTCTAC	5′CATGTTCACTTCGTCCTCAGA	NM_174425.2
PLCZ1	Phospholipase C zeta-1	5′GTCGGAATCCCACTCTTCAG	5′GGCCACCATTTGACAACCTA	NM_001011680.2
PTGFR	*Bos taurus* prostaglandin F receptor (FP)	5′GGGGCACTTTTGTCAGACTTGGC	5′AGGCCCAAACTGCTTTCGTTTGA	NM_181025.2
Housekeeping gene				
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	5′AATATCATCCCTGCTTCTACTGG	5′CATACTTGGCAGGTTTCTCCA	NM_001034034.1

of cDNA (BioRad iQ5 Thermal Cycler; BioRad). Each sample was run in duplicate, and real-time reactions were repeated at least twice. The parameters for PCR were an initial RT inactivation and Taq polymerase activation step at 94°C for 3 min, denaturation at 94°C for 30 sec, annealing at 60°C for 30 sec (for all primer sets except RYRs at 59°C and ORAI at 55°C), and extension at 72°C for 1 min. Forty cycles were used for each primer set.

For each gene expressed in CL, melt curve analysis revealed amplification of a single gene product. Validation of a single gene product was performed by 1) analyzing a single peak in the melt curve and 2) visually by running PCR products on a 2% agarose gel (data not shown). Threshold cycle (Ct) values were obtained and used in the mathematical model described by Pfaffl [37] for semiquantitation after normalization with the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Previous studies from our laboratory [38, 39] and other investigators [40] validated GAPDH as a normalizing standard to assess gene expression at different luteal developmental stages. If primers were unable to amplify a signal for the corresponding gene, indicating that those genes may not expressed in bovine CL, the primers were tested further by verifying that they were able to produce a single gene product when using total RNA isolated from a positive control of bovine cerebellum or uterine tissues (data not shown). All real-time PCR assays were repeated at least twice for quality analysis and were determined to produce similar results. For the comparison between developing and mature corpora lutea, the Day 4 mRNA expression value was set as the control and compared to mRNA isolated from Day 10 samples.

Semiquantitative RT-PCR for Sorcin

Six primer sets for the sorcin gene (SRI; designed via NCBI, PrimerBlast, http://www.ncbi.nlm.nih.gov/tools/primer-blast) were tested for reliability. All tested primer sets for bovine sorcin gene expression were inefficient at yielding one gene product as revealed by the melt curve in real-time PCR and on 2% agarose gel. Therefore, only one primer set was chosen. The SRI PCR product from this primer set, size of 156 bp, was isolated and sequenced to confirm the sorcin gene (WVU Genomics Sequencing Core). Although real-time PCR could not be implemented, semiquantitative PCR was carried out as previously described [39], with slight modifications. Briefly, cDNA from developing (Day 4) and mature (Day 10) CL was amplified with SRI and GAPDH (housekeeping gene) primers (Table 1) via PCR (Invitrogen, Carlsbad, CA). The parameters for PCR were an initial RT inactivation and Taq polymerase

activation step at 95°C for 3 min, denaturation at 95°C for 60 sec, annealing at 58°C for 60 sec, and extension at 72°C for 1 min for 35 cycles. PCR products were run in duplicates on a 2% agarose gel. All Day 4 samples were run together, as were Day 10 samples, to minimize inherent error within the gel. Images of the bands were captured using the AlphaImager. Integrated density values (IDVs) were calculated using densitometry Alpha View Software for Fluor Chem Systems. Average IDVs were ascertained and used to quantify the expression of each gene.

Semiquantitative Western Blotting

Sample proteins were analyzed using semiquantitative Western blotting as previously described [41]. Briefly, protein samples from Day 4 (n = 3; n = 4 for PLCB1) and Day 10 (n = 3; n = 4 for PLCB1) CL were obtained by pulverizing and homogenizing the tissue in a buffer containing 50 mM Tris HCl, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 0.1% SDS, 1% Triton-X, protease inhibitors (2 mM phenylmethylsulfonylfluoride, 5 μg/μl leupeptin, 5 μg/μl aprotinin), and phosphatase inhibitors (100 mM sodium fluoride, 2 mM sodium orthovanadate, 10 mM sodium pyrophosphate). Protein concentration in samples was determined by the Bio-Rad assay with bovine serum albumin (BSA; Gibco BRL) as the standard; 180 μg/lane of protein were loaded for PLCB1 and RYR2, 100 μg/lane for ITPRs, and 75 μg/lane for ATP2A2 and SRI. After polyacrylamide gel (8% or, in the case of RYR2, 6%) electrophoresis separation, the proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (Perkin Elmer) as previously described [29].

Primary antibodies used included 1) mouse monoclonal to PLCB1 (ab77743) at a dilution of 1:200 (v/v) (Abcam), 2) mouse monoclonal to RYR2 (ab2868) at a dilution of 1:5000 (v/v) (Abcam), 3) goat polyclonal to ITPR2 (sc-26386) at a dilution of 1:200 (v/v) (Santa Cruz Biotechnology), 4) goat polyclonal to ITPR3 (sc-7277) at a dilution of 1:2000 (v/v) (Santa Cruz Biotechnology), 5) mouse monoclonal to SERCA2 (ATP2A2) (ab2817; Abcam) at a dilution of 1:500 (v/v) (Abcam), 6) mouse monoclonal to PMCA (ATP2B) (ab2825; Abcam) at a dilution of 1:250, 7) mouse monoclonal to beta actin (AM4302; Ambion Life Technologies) at a dilution of 1:2000 (v/v), and 8) rabbit polyclonal to SRI (ab71983; Abcam) at a dilution of 1:500 (v/v) overnight at 4°C with gentle shaking. The secondary horseradish peroxidase-conjugated antibodies or infrared fluorophore dye-conjugated antibodies used were: 1) goat anti-mouse antibody (for actin, RYR2, and PLCβ1) diluted to 1:10 000 (v/v) (Jackson Immunoresearch) 2) donkey anti-goat (for ITPR2 and ITPR3) diluted to 1:2000 (v/v) (Abcam),3) goat anti-mouse IRDye 680RD (for actin and ATP2A2) diluted to 1:10 000, and 4) goat anti-rabbit IRDye 800CW (for SRI) diluted to 1:10 000 (v/v) (Amersham Life Sciences). Intensity of the bands of interest was captured by film or Odyssey infrared imaging software and quantified through densitometry (AP Fluor software, Alpha Innotech, or Odyssey Infrared Imaging System) The signal intensity for the protein of interest was standardized to the corresponding intensity of the actin control in that sample. This normalization allowed for the semiquantitative estimation of the amount of protein in the samples of interest, as determined previously [41].

Immunohistochemistry

Samples from four Day 4 and four Day 10 CL were placed into chilled saline solution immediately on collection. Tissue processing was performed as previously described [36] with minor modifications. Briefly, tissue samples were fixed for at least 48 h in Bouin fixative. The fixative was washed by repetitive immersion in 70% ethanol. Subsequently, the tissue was dehydrated via successive 95% and 100% ethanol. Luteal tissue was cleared by submersion in D-limonene clearant (Fisher Scientific). Subsequently, the cleared tissue samples were embedded by immersion in paraplast in preparation for sectioning and mounting the sections on microscope slides.

For sorcin, immunofluorescence detection was used, and all steps for this procedure were carried out at room temperature in a humidified light-tight box unless otherwise noted. Sections were treated with normal goat serum (5% NGS) to reduce nonspecific binding. The primary antibody consisted of rabbit polyclonal to SRI (Rb pAB, ab71983; Abcam) at a concentration of 10 μg/ml. The secondary antibody consisted of Texas Red conjugated goat anti-rabbit IgG at concentration of 10 μg/ml in 0.1% NGS-Tris buffer. Each immune detection included two negative controls, one slide on which primary antibody was substituted by normal rabbit serum (10 μg/ml in 0.1% NGS-Tris buffer) and another on which secondary antibody was omitted. Samples were incubated overnight at 4°C while in the primary antibody. The following day, tissue sections were washed with Tris buffer and then treated with secondary antibody in complete darkness. The secondary antibody was removed, and the tissue was further washed three times with Tris buffer prior to the application of coverslips. Mounted samples were examined under an Olympus PRO-VIS AX70 (Olympus America) upright fluorescence microscope for analysis of tagged SRI within steroidogenic cells based on quantifiable Texas Red fluorescence. This method of fluorescent staining was chosen, as this very specific spectrum allowed for isolation of the SRI protein while diminishing autofluorescence. Specific investigation of corpora lutea for fluorescent intensity was limited to steroidogenic cells, with particular focus on LLCs.

For specific detection of SERCA and RyR on Day 4 and Day 10 luteal samples, prepared as described above, an immunoenzymatic antigen detection system from Abcam (ab80436) was utilized following the manufacturer's protocol provided with the IHC kit. The same primary antibodies described in the WB section were used in this protocol. Serca antibody (ab2817) and RyR antibody (ab2368) were applied overnight at 1:100 and 1:50 dilution, respectively. Effective detection of these two proteins required heat-induced antigen retrieval with sodium citrate buffer. Deparafinized sections were incubated at 98°C for 20 min in sodium citrate buffer (10 mM sodium citrate, 0.05% Tween 20, pH 6.0). Negative controls, that is, the omission of primary and/or secondary antibodies, were included to validate this HRP/DAB detection procedure. Detection was performed in duplicate sections prepared from CL obtained from four Day 4 and four Day 10 cows. Samples were examined and photographed under an Olympus PRO-VIS AX70 microscope.

Phospholipase C Activity Assay: Determination of IP1 Concentration

Because IP3 is metabolized quickly (half-life of IP3 is approximately 1 sec) into inositol bisphosphate (IP2) and inositol monophosphate (IP1), IP1 accumulation can be used as an indicator of IP3 generation from PIP_2 degradation via phospholipase C. IP1 concentrations were measured in enriched luteal steroidogenic cells after stimulation by media alone (control) or PGF2α. Enriched luteal steroidogenic cells were isolated as previously described [19]. Enriched steroidogenic luteal cells from Day 4 (n = 2) and Day 10 (n = 3) were plated in 384-well white microplates (Greiner Bio One) with 30 μl of M199 media containing 10% FBS and 1% penicillin/streptomycin. Luteal cells were incubated overnight at 37°C in a CO_2 atmosphere. The IP-One Tb assay from Cisbio was employed to measure IP1 concentration, according to the manufacturer's instructions. Briefly, after an overnight incubation, the cell supernatant was removed. Ten microliters of the IP1 stimulation buffer (10 mM Hepes, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 4.2 mM KCl, 146 mM NaCl, 5.5 mM glucose, 50 mM LiCl, pH 7.4) were added to negative controls, and PGF2α-containing stimulation buffer was added to the positive group cells. Number of cells, PGF2α concentration, and incubation time were optimized for this assay. Cells were incubated at 37°C in a CO_2 atmosphere for 30 min. After incubation, the plates were read on a Synergy 2 spectrophotometer. Two sequential measurements at 620 and 665 nm were taken to determine IP1 concentration. The mean and standard deviation were calculated for each replicate. The delta F was calculated for interassay reproducibility and found to be 80% to 90% similar. The coefficient of variation for the intra-assay duplication was between 0.1% and 6%.

Determination of the Calcium Source for the PGF2α-Stimulated Rise in $[Ca^{2+}]i$ in Cells Isolated from Developing and Mature Bovine CL

Intracellular calcium concentration. Dispersed total luteal cells isolated from developing and mature CL were used to determine the calcium source and the effect of pharmacological agents on PGF2α-induced rise in $[Ca^{2+}]_i$ using FURA-2 dye (Calbiochem), a modified method previously described [19]. Dispersed total luteal cells were counted with a hemocytometer, and the density was adjusted to 1×10^5 cells/ml by adding bicarbonate-buffered M199 supplemented with 5.0% fetal calf serum (FCS). This initial concentration of FCS in the M199 allows luteal cell attachment to the microscope slides. An 80-μl aliquot of the cell suspension was applied to a Cunningham chamber constructed on poly-L-lysine-coated microscope slides [19]. The Cunningham chambers were maintained for 6 h in a humidified incubator (37°C, 95% air-5% CO_2). Poly-L-lysine, Medium 199, FCS, and penicillin-streptomycin were from Life Technologies.

After the 6-h attachment period, the cells were used for calcium measurements. The experimental tissue culture media in these experiments consisted of 127 mM NaCl, 5 mM KCI, 1.8 mM $CaCl_2$, 2 mM $MgCl_2$, 5 mM $KHP0_4$, 5 mM $NaHC0_3$, 10 mM HEPES, 10 mM glucose, and 0.1% BSA, pH 7.4. Calcium-free medium was prepared by adding 2.5 mM EGTA to the above experimental media. Luteal cells were loaded with 1 μM fura-2/AM (Calbiochem) in experimental medium (serum free, containing calcium, and without hormones) for 1 h at 37°C. The cells were washed with experimental

TABLE 2. Expression of mRNA in developing and mature bovine corpora lutea.

Gene symbol[a]	Day 4 (mean ± SEM)	Day 10 (mean ± SEM)	*P*-value, *t*-test	*P*-value, Wilcoxon test
Components of the endoplasmic reticulum				
ATP2A2	1.02 ± 0.12	0.49 ± 0.11	0.01*	
ITPR2	1.05 ± 0.18	2.69 ± 1.08	0.11	
ITPR3	1.02 ± 0.13	2.19 ± 1.48	0.25	
RYR2	1.30 ± 0.51	12.86 ± 4.36	0.04*	
RYR3	1.52 ± 0.83	15.29 ± 8.79		0.13
SRI	0.17 ± 0.01	0.17 ± 0.02	0.49	
Components of the plasma membrane				
ATP2B1	1.09 ± 0.25	4.51 ± 0.81	0.01*	
ATP2B3	1.17 ± 0.32	2.81 ±1.25	0.12	
ATP2B4	1.00 ± 0.05	15.82 ± 8.15	0.09	
CACNA1C	1.60 ± 0.99	2.55 ± 0.51	0.44	
CACNA1B	1.13 ± 0.31	2.54 ± 2.12		0.75
ORAI2	1.38 ± 0.55	0.59 ± 0.19	0.11	
PLCB1	1.62 ± 0.98	2.07 ± 0.87	0.37	
PTGFR	1.06 ± 0.22	0.55 ± 0.20	0.07	

[a] *ATPA2*, calcium transporting, cardiac muscle, slow twitch 2 (SERCA2); *ITPR2*, inositol 1,4,5-trisphosphate receptor, type 2; *ITPR3*, inositol 1,4,5-trisphosphate receptor, type 3; *RYR2*, ryanodine receptor type 2; *RYR3*, ryanodine receptor type 3; *SRI*, sorcin.
* Indicates a significant difference.

medium containing or lacking calcium to ascertain the source of calcium during treatment with PGF2α, which was prepared in media containing or lacking calcium, and incubated for an additional 30 min at 37°C to allow cytoplasmic de-esterification of the fura-2/AM dye.

After fura dye loading, the Cunningham chamber was placed on the stage of an Olympus PRO-VIS AX70 microscope (Olympus America) equipped for epifluorescent microscopy. All experiments were performed at room temperature (22–25°C). The dual wavelength images were acquired and analyzed using the ratio/fret/densitometry module of SlideBook 4.1 software for Windows (Intelligent Imaging Innovations, B&B Microscopes). The luteal cells were challenged with PGF2α at a concentration of 1 μg/ml, which had been previously demonstrated to elicit maximal responses in cells isolated from both developing and mature CL [19]. The PGF2α challenge also was performed in the presence of 200 nM ryanodine with or without calcium. The fluorescence ratio data were used to calculate the area under the curve of the elicited responses.

Luteal Cell Dispersion and Enrichment

Luteal cell dispersion was performed as previously described [19]. Briefly, the CL tissue was dissociated in cell dispersion medium (CDM, M-199 containing 0.1% BSA, 25 mM Hepes, 100 U/ml fungicide) containing collagenase type IV (420 U/ml/g of tissue; GIBCO, Invitrogen Life Technologies). The dispersed luteal cells were then suspended in 1% PBS, mixed with magnetic tosylactivated beads (Dynal Biotech), coated with BS-lectin, and placed for 25 min at 4°C on a rocking platform. The bead-adherent cells were separated using a magnetic particle concentrator (Dynal Biotech). Nonadherent cells (steroidogenic luteal cells) were then collected, washed with 1% PBS, and lysed to prepare protein samples as described under *Semiquantitative Western Blotting* or cultured for determination of PLC activity.

Statistical Analysis

Expression ratios of mRNA and protein between Day 4 and Day 10 CL were analyzed as indicated below. Protein and mRNA expression data were tested for normal distribution via the Shapiro-Wilk test. The one-tailed Student *t*-test was used for comparison of data sets that had a normal distribution. For data sets that did not have a normal distribution, a one-tailed Wilcoxon two-group test (Mann-Whitney test) was used [42]. The one-tailed Student *t*-test was used to compare Day 4 and Day 10 IP1 concentrations. The areas under the curve corresponding to the calcium responses were analyzed using a two-way ANOVA with Tukey HSD post hoc test. All tests were conducted using the JMP software version 9.0 (SAS Institute). Statistical significance was set at $P < 0.05$. Data in all graphs are depicted as the mean ± SEM.

RESULTS

Defining the Major Genes Participating in Calcium Homeostasis in the Bovine CL

The expression on mRNA in developing and mature bovine corpora lutea is summarized in Table 2. Data are presented as means ± SEM and *P*-values (Student *t*-test and Wilcoxon test). An asterisk indicates significance.

Endoplasmic reticulum-related genes involved in calcium homeostasis. Expressed mRNAs in this category were one ER calcium pump, *ATP2A2*; two IP3 receptors-calcium release channels, *ITPR2* and *ITPR3*; and two ryanodine receptor-calcium release channels, *RYR2* and *RYR3* (Table 2). In addition, an ER-closely associated gene, Sorcin (*SRI*), was expressed in bovine CL (Table 2). Although mRNAs encoding *ITPR1* and *RYR1* were not detected in bovine CL, the utilized primers effectively amplified mRNAs corresponding to these genes in bovine cerebellum and uterus, respectively (data not shown).

Plasma membrane-related genes involved in calcium homeostasis. Expressed mRNAs in this category were three calcium ATPase pumps, *ATP2B1*, *ATP2B3*, and *ATP2B4*; two voltage-gated calcium channels, *CACNA1C* and *CACNA1B*; one store-operated calcium channel, *ORAI2*; the phospholipase beta enzyme *PLCB1*; and the receptor for PGF2α, (*PTGFR*) mRNA. All were expressed in bovine CL (Table 2). Messenger RNAs encoding *ATP2B2*, *CACNA1H*, *ORAI1*, *ORAI3*, *PLCδ3*, *PLCγ1*, and *PLCζ1* were not expressed in bovine CL but were expressed in bovine cerebellum (data not shown).

Expression of Genes Involved in Calcium Homeostasis as a Function of Development

Endoplasmic reticulum-related genes involved in calcium homeostasis. Expressions of mRNA encoding *ITPR2*, *ITPR3*, *RYR3*, and *SRI* were not statistically different as a function of luteal development (Table 2). In contrast, expression of *ATP2A2* was decreased from developing (Day 4; 1.02 ± 0.12) to mature (Day 10) CL (0.49 ± 0.11, $P = 0.01$; Table 2). Expression of *RYR2* was increased from developing (1.30 ± 0.51) to mature CL (12.86 ± 4.36, $P = 0.04$; Table 2).

TABLE 3. Western blotting assessment of protein expression of genes involved in calcium homeostasis as a function of development.

Protein symbol[a]	Day 4 (means ± SEM)	Day 10 (means ± SEM)	*P*-value, *t*-test
Components of the endoplasmic reticulum			
ATP2A2	0.14 ± 0.05	0.02 ± 0.01	0.04*
ITPR2	2.25 ± 0.16	1.87 ± 0.09	0.06
ITPR3	2.53 ± 0.18	2.13 ± 1.48	0.07
RYR2	0.32 ± 0.01	0.72 ± 0.04	0.03*
SRI	0.4 ± 0.07	0.03 ± 0.02	0.005*
Components of the plasma membrane			
PLCB1	0.67 ± 0.01	0.67 ± 0.03	0.48

[a] ATPA2, calcium transporting, cardiac muscle, slow twitch 2 (SERCA2); ITPR2, inositol 1,4,5-trisphosphate receptor, type 2; ITPR3, inositol 1,4,5-trisphosphate receptor, type 3; RYR2, ryanodine receptor type 2; SRI, sorcin.
* Indicates a significant difference.

Protein expression was analyzed for ITPR2, ITPR3, RYR2, ATP2A2, and SRI (Table 3). Protein expressions of ITPR2 and ITPR3 corroborated mRNA expression, indicating that the difference between developing and mature CL was not significant. Representative images of Western blot for ITPR2 and ITPR3 are shown in Figure 2. RYR2 protein expression from whole CL samples supported mRNA expression, demonstrating an increase from developing (0.32 ± 0.01) to mature CL (0.72 ± 0.04, $P = 0.03$; Table 3 and Fig. 3A). ATP2A2 protein expression from whole CL samples supported mRNA expression with a decrease from developing (0.14 ± 0.05) to mature CL (0.02 ± 0.01, $P = 0.04$; Table 3 and Fig. 3B). SRI protein expression was decreased in mature (0.03 ± 0.02) compared to developing CL (0.4 ± 0.07, $P = 0.0056$; Table 3 and Fig. 3B).

As protein expression for RYR2, ATP2A2 (SERCA), and SRI (sorcin) examined in samples prepared from whole CL was affected by development, their expression was further investigated in samples prepared from enriched steroidogenic luteal cells. The difference in expression for RYR2 protein in enriched steroidogenic cells isolated from developing CL (4.2 ± 0.4, n = 4) or mature CL (3.1 ± 0.4, $P = 0.3$, n = 4; Fig. 4A) was not significant. ATP2A2 protein expression in enriched steroidogenic cells decreased from developing (6.5 ± 0.4) to mature CL (3.7 ± 0.4, $P = 0.01$, n = 4; Fig. 4B). SRI protein expression was decreased in enriched steroidogenic cells from mature CL (3.3 ± 0.1) compared to developing CL (4.7 ± 0.1, $P = 0.07$, n = 4; Fig. 4A).

Plasma membrane-related genes involved in calcium homeostasis. The mRNA encoding components of the plasma membrane, *ATP2B1*, *ATP2B3*, *ATP2B4*, *CACNA1C*, *CACNA1B*, *ORAI2*, *PLCB1*, and *PTGFR* did not change expression from Day 4 to Day 10 (Table 2). In contrast, the expression of mRNA for *ATP2B1* increased from Day 4 (1.09 ± 0.25) to Day 10 (4.51 ± 0.81, $P = 0.01$; Table 2).

Protein expression was analyzed for PLCB1 (Table 3). PLCB1 protein expression supported the mRNA data, indicating that the difference between Days 4 and 10 was not significant.

SRI Immunohistochemistry

A total of 806 steroidogenic cells from five developing (Day 4) and five mature (Day 10) corpora lutea were analyzed for quantification of immunofluorescence (Fig. 5A). Cells included in this quantification were defined to be clearly discernible with a lucid nuclear outline and very clear cellular boundary. The average fluorescence of steroidogenic cells in developing and mature CL were 1600 ± 35.39 and 1461 ± 10.21, respectively

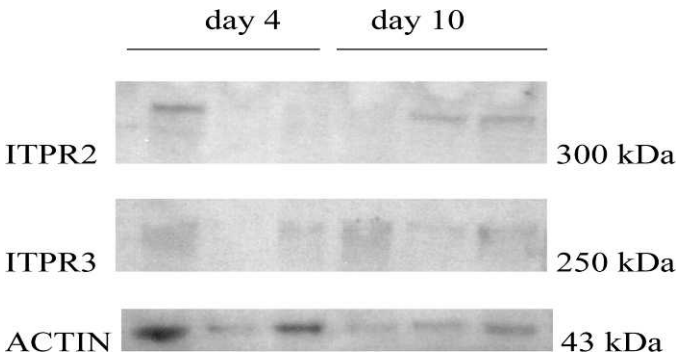
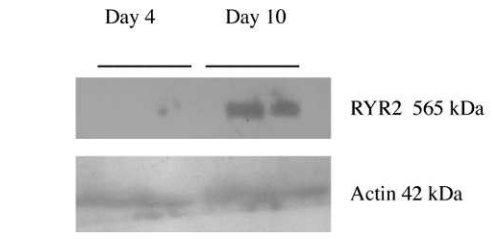


FIG. 2. Representative Western blot showing amounts of ITPR2 (inositol 1,4,5-trisphosphate receptor, type 2), ITPR3 (inositol 1,4,5-trisphosphate receptor, type 3), and actin expressed in total protein samples from bovine Day 4 (developing) and Day 10 (mature) CL.



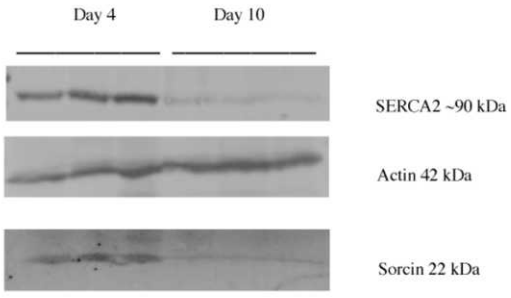


FIG. 3. Representative Western blot showing amounts of RYR2 (ryanodine receptor, type 2; **A**), SERCA (ATP2A2, calcium transporting, cardiac muscle, slow twitch 2; **B**), sorcin, and actin expressed in total protein samples from bovine Day 4 (developing) and Day 10 (mature) CL.

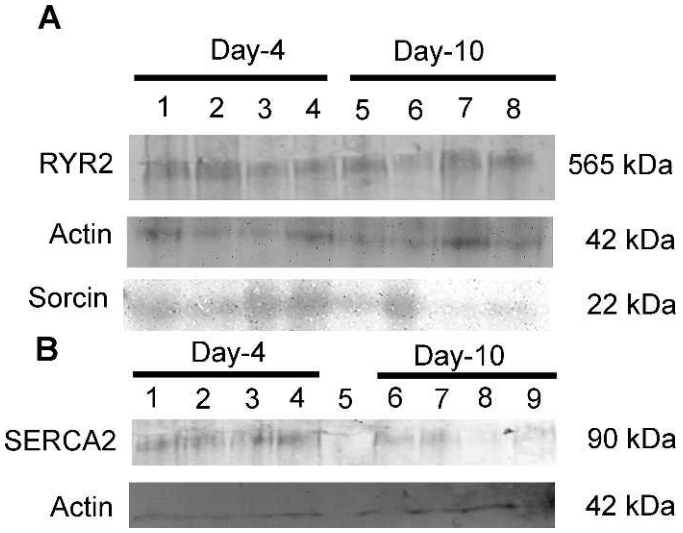


FIG. 4. Representative Western blot showing amounts of RYR2 (ryanodine receptor, type 2; **A**), actin, and sorcin (SRI) and SERCA2 (ATP2A2, calcium transporting, cardiac muscle, slow twitch 2; **B**) and actin, expressed in protein samples from enriched steroidogenic cells isolated from Day 4 (developing, lanes 1–4, **A** and **B**) and Day 10 (mature, lanes 5–8, **A**; lanes 6–9, **B**) CL.

(Fig. 5B). The difference in mean fluorescence for the two developmental stages was statistically significant ($P = 0.0001$; Fig. 5C). The decrease in SRI protein by immunohistochemical assay corroborated the Western blot analysis.

Luteal Cellular Localization of ATP2A2 (SERCA) and RYR2

Five hundred cells each identified as large and small luteal cells were documented to display specific immunoreactivity for ATP2A2 and RYR2 at both developmental stages examined (panels A and B in Figs. 6 and 7, respectively). Attempts were made to use immunofluorescence assay for detecting cellular

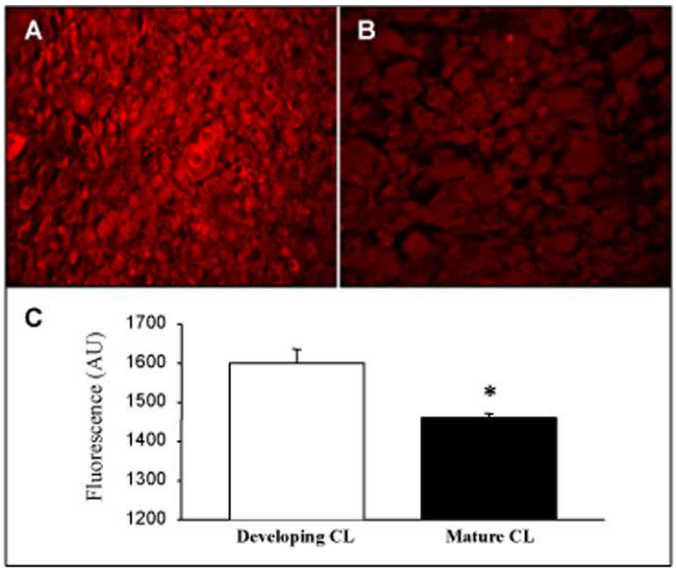


FIG. 5. Immunofluorescent images of sorcin captured by an Olympus Provis fluorescent microscope at ×60 magnification of a Day 4 (developing, **A**) and a Day 10 (mature, **B**) CL. The average fluorescence of LLCs within developing CL was 1600 units and in a mature CL was 1461 units (**C**). Average fluorescent units (± SEM) of sorcin in developing and mature CL. *Denotes significance at $P < 0.05$.

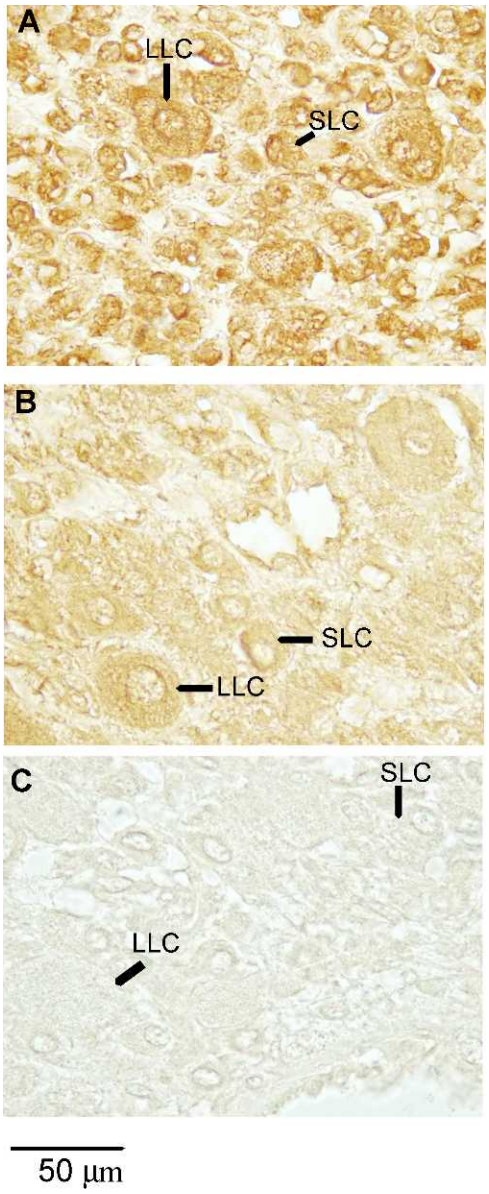


FIG. 6. Specific detection of SERCA2 (ATP2A2, calcium transporting, cardiac muscle, slow twitch 2) in steroidogenic bovine luteal cells by immunohistochemistry. **A**) Representative immunoreactivity observed in large (LLC) and small (SLC) steroidogenic cells in a section of Day 4 (developing) CL. **B**) Immunoreactivity detected in large (LLC) and small (SLC) steroidogenic cells in a section of Day 10 (mature) CL. **C**) Specificity of the assay by a dramatic reduction of immunoreactivity in both cell types of a Day 4 CL when the primary antibody was omitted. Bar = 50 μm.

location of ATP2A2 and RYR2, but in our hands, these antibodies did not yield satisfactory results. Effective detection of these two proteins required heat-induced antigen retrieval followed by an immunoenzymatic antigen detection system. Large and small steroidogenic cells were easily identified based on morphology and size. Examples of these cells are shown in panels A, B, and C in Figures 6 and 7. Immunoreactivity was specific as demonstrated by its elimination when primary antibodies (panel C in Figs. 6 and 7) or secondary antibodies (not shown) were eliminated from the assay. For ATP2, immunoreactivity appeared to be cytoplasmic in location, and although no attempt at quantitative assessment was made, it appeared to be consistently of greater intensity in samples from developing than mature CL (panels A and B in Fig. 6). For

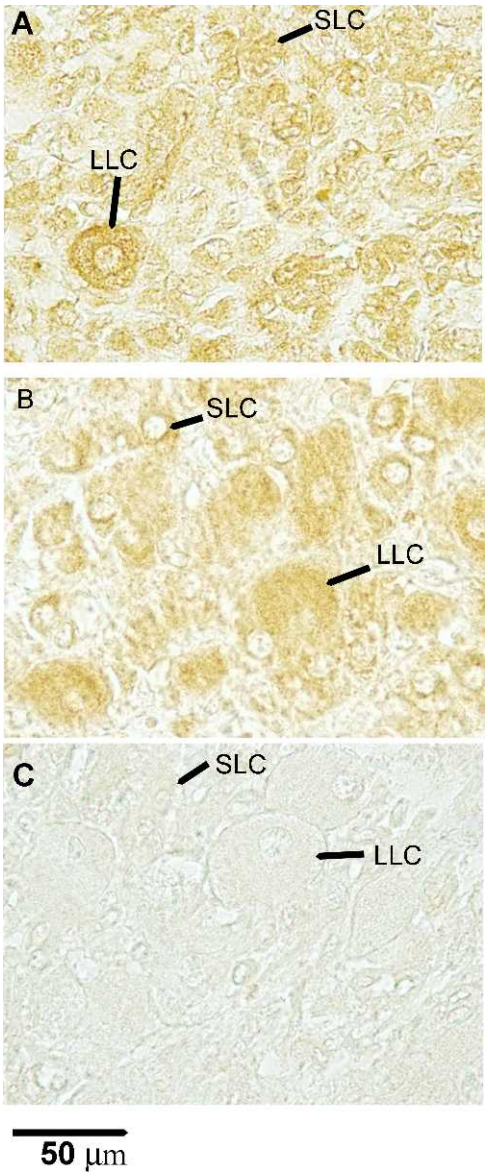


FIG. 7. Specific detection of RYR2 (ryanodine receptor, type 2) in steroidogenic bovine luteal cells by immunohistochemistry. **A**) Representative immunoreactivity observed in large (LLC) and small (SLC) steroidogenic cells in a section of Day 4 (developing) CL. **B**) Immunoreactivity detected in large (LLC) and small (SLC) steroidogenic cells in a section of Day 10 (mature) CL. **C**) Specificity of the assay by a dramatic reduction of immunoreactivity in both cell types of a Day 10 CL when the primary antibody was omitted. Bar = 50 μm.

RYR2, immunoreactivity also appeared to be cytoplasmic in location, and no apparent difference in intensity was discernible at the two luteal developmental stages examined (panels A and B in Fig. 7). Additional RYR2 immunoreactivity was documented in luteal vasculature, especially in cells of the tunica media and intima of large vessels (data not shown). It was very difficult to assess ATP2A2 or RYR2 immunoreactivity in endothelial cells of capillaries due to the very delicate amount of cytoplasm of cells lining these vessels.

Determination of Phospholipase C Activity via IP1 Concentration

Optimal number of luteal cells was determined to be 20 000 cells per well. PGF2α optimal concentration for 20 000 cells was determined to be 5.0 ng/μl. The average IP1 concentration increased from 27.7 ± 12.7 nM in developing luteal cells to 326. 4 ± 41.2 nM in mature luteal cells on PGF2α stimulation ($P = 0.009$; Fig. 8) The average IP1 concentration for media (IP1 buffer) only was 15 ± 4 nM in developing luteal cells and 60.6 ± 5.8 nM in mature luteal cells.

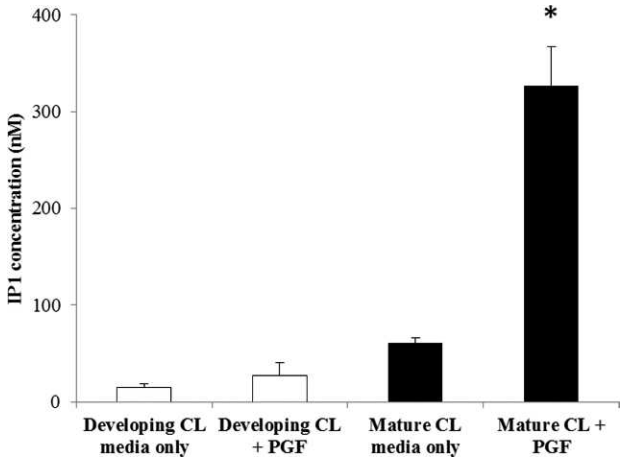


FIG. 8. Measurement of PLC activity by determination of IP1 concentration after stimulation with 5.0 ng/μl PGF2α. In media only, IP1 concentration in Day 4 (developing) was 15 ± 4 nM, and Day 10 (mature) CL was 60.6 ± 5.8 nM. For PGF2α-stimulated luteal cells, IP1 concentration in developing CL was 27.7 ± 12.7 nM and in mature CL was 326.4 ± 41.2 nM. *Denotes significance at $P < 0.05$.

Determination of the Calcium Source for the PGF2α - Stimulated Rise in $[Ca^{2+}]_i$ in the Developing and Mature Bovine Large Luteal Cells

Removing extracellular calcium affected the response elicited by PGF2α to increase $[Ca^{2+}]_i$ in LL cells isolated from developing ($P = 0.02$; Fig. 9) but not from mature CL. Similarly, an effect of ryanodine in reducing the response elicited by PGF2α on increasing $[Ca^{2+}]_i$ was demonstrated only in cells isolated from mature CL ($P = 0.03$, with calcium, and $P = 0.047$, without calcium; Fig. 9).

DISCUSSION

Results clearly indicate that in bovine CL, there are at least four ER components with the ability to contribute to an increase in $[Ca^{2+}]_I$; two inositol 1,4,5-trisphosphate receptors, ITPR2 and ITPR3; and two ryanodine receptors, RYR2 and RYR3. In addition, the calcium-binding protein sorcin (SRI) could potentially associate with RYRs, which could further modulate calcium release from the ER. In samples prepared from whole CL, relative amounts of both mRNA and protein corresponding to *RYR2* were increased in mature compared to developing CL. However, when RYR2 protein was assessed in samples from enriched luteal steroidogenic cells, there was no difference associated with luteal development. Therefore, differential expression of RyR2 detected in whole CL must be contributed by a luteal compartment other than steroidogenic cells. Likely contributors to this difference are cells from tunica media and intima of large blood vessels, as specific RYR2 immunoreactivity was documented there (data not shown). Although amount of RYR2 protein per se does not appear to be part of the cellular basis accounting for luteal developmental difference in calcium response to exogenous PGF2α, its functional status might be part of it. This is so

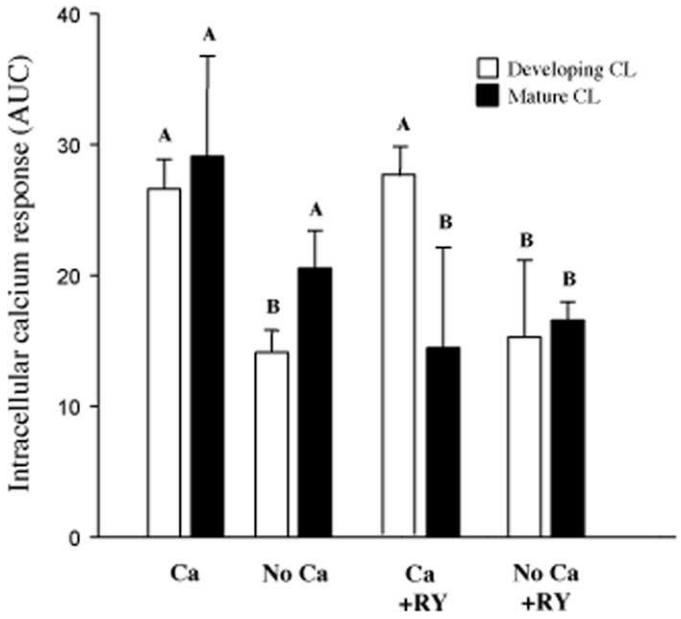


FIG. 9. Intracellular calcium ion response in bovine large luteal cells on PGF2α stimulation (1.0 μg/ml). The stimulation was performed in the presence (Ca) or absence (No Ca) of extracellular calcium (Ca^{2+}), in the presence of extracellular Ca^{2+} with ryanodine (Ca+RY), and in the absence of Ca^{2+} with ryanodine (No CA+RY). Different letters denote significance of $P < 0.05$.

because another component of the cellular mechanism appears to be posttranslational regulation of SRI such that the relative amount of SRI protein but not mRNA was greater in developing than in mature CL. This interpretation is supported strongly by the observation that this was found both in samples from whole corpora lutea and in enriched luteal steroidogenic cells (Table 3 and Figs. 3B and 4A). Furthermore, greater gene expression encoding the ER Ca^{2+}-ATPase pump (ATP2A), responsible for calcium reuptake into the ER, in developing than in mature CL means that developing CL have greater calcium buffering capacity. This pattern of ATP2A expression is likely to be part of the cellular mechanism partially responsible for the developmental difference in calcium response to exogenous PGF2α, as difference in expression was found in both whole corpora lutea and enriched luteal steroidogenic cells (Table 3 and Figs. 3B and 4B). Another component of calcium homeostasis in steroidogenic cells that is likely to explain the developmental difference in calcium response to exogenous PGF2α is the greater PLCβ activity observed in those cells isolated from mature CL. Greater generation of IP3 would more effectively release calcium from the ER via unchanging amounts of the expressed receptors, ITPR2 and ITPR3.

The patterns of expression observed for three plasma membrane Ca^{2+}-ATPases: ATP2B1, ATP2B3, and ATPB4; the two voltage-activated calcium channels: L-type CACNA1C and N-type CACNA1B; one calcium release-activated channel: ORAI2; and the PGF2α receptor PTGFR (Table 2) do not appear to be part of the mechanism that explains the developmental difference in calcium response to exogenous PGF2α.

IP3 is generated by the cleavage of phosphatidylinositol 4,5-bisphosphate (PIP2) by phospholipase C (PLC). In bovine [43] and primate [23] corpora lutea, there is strong evidence that PGF2α activates PLC on binding to its receptor in steroidogenic cell. The data herein reveal that the specific isotype of PLC, PLCβ1, was present and that its expression was not different in the developing and mature bovine CL. Moreover, it was confirmed (Table 2) that expression of the PGF2α receptor (PTGFR) in the bovine CL is not developmentally regulated, as previously described [13]. Although gene expression of PLCβ1 during development was not different, PLC enzyme activity, indicated by measurement of IP1, was greater in sterodogenic cells isolated from the mature compared to the developing CL. Previous studies determined that PLC was stimulated in mature bovine CL by PGF2α in both large and small luteal cells [17]. The increase in phospholipase C activity in mature CL may be due to changes in Ca^{2+} binding, phosphorylation or amount of substrate, PIP_2 [44–47]. In addition, an increase in $[Ca^{2+}]_i$ in the mature CL and the activation of phosphorylating enzymes, such as PKC, may alter the activity of PLCβ1 at this stage [48]. Further investigations to examine these possible mechanisms will be necessary to confirm how this enzyme is activated.

Expression of ITPR2 and ITPR3 was detected in both developing and mature bovine corpora lutea in the present investigation. In mice, only ITPR2 was detected in CL, but luteal developmental stage was not reported [49]. In pigs, ITPR2 was reported to be expressed in developing but not mature CL [50]. It is not clear if this is due to species differences or if the results reflect other technical or aspects of luteal physiology. Protein and mRNA expression encoding the ITPRs did not differ between developing and mature bovine CL in this study. However, the possibility that functional aspects of ITPRs, such as phosphorylated state, cytosolic Ca^{2+} availability, and the concentration of inositol 1,4,5-trisphosphate (IP3) [51], contribute to differences in calcium homeostasis at these two luteal developmental stages cannot be ruled out. Indeed, greater activity of PLC in the mature CL, which results in an increase in IP3 released, most likely mediates an increase in the opening probability of the ITPRs, leading to a greater magnitude of the rise in $[Ca^{2+}]_i$.

Coordinated mechanisms for increasing $[Ca^{2+}]_i$ via CICR have been documented in reproductive and nonreproductive tissues. In mouse luteinized-granulosal cells, both RYRs and ITPRs were detected, although specific isotypes were not identified [30]. These authors suggested that RYRs might have a functional association with ITPRs and that these channels could participate in a coordinated fashion via a CICR mechanism to increase $[Ca^{2+}]_i$ [30]. This type of interdependent action has been described in rat gastric myocytes [52], vas deferens myocytes [53], vagal sensory neurons [54], and mouse Leydig cells [29]. The present data revealed that protein expression of sorcin (SRI), a calcium-binding protein that binds specifically to RYRs [55], was decreased in the mature CL as compared to the developing CL. A reduction in SRI results in decreased binding to inhibit RYRs, therefore allowing more Ca^{2+} to exit the ER. These results indicate that the mature CL acquires additional functional mechanisms for releasing Ca^{2+}, thereby increasing $[Ca^{2+}]_i$.

The function of the ER Ca^{2+}-ATPase pump, ATP2A, is to exchange two cytosolic Ca^{2+} ions into the ER for two or three intraluminal H^+ ions outside the ER per one ATP hydrolyzed [56]. If, as analyses of the present data indicate, the increased gene and protein expression of ATP2A2 in the developing CL is associated with greater calcium uptake into the ER, then mature CL has not only additional mechanisms for calcium release than developing CL but also less capacity for calcium reuptake. It is proposed that these cellular mechanisms contribute to the ability of PGF2α to stimulate an increase in $[Ca^{2+}]_i$ of greater magnitude in mature than in developing bovine CL. It is therefore postulated that these luteal

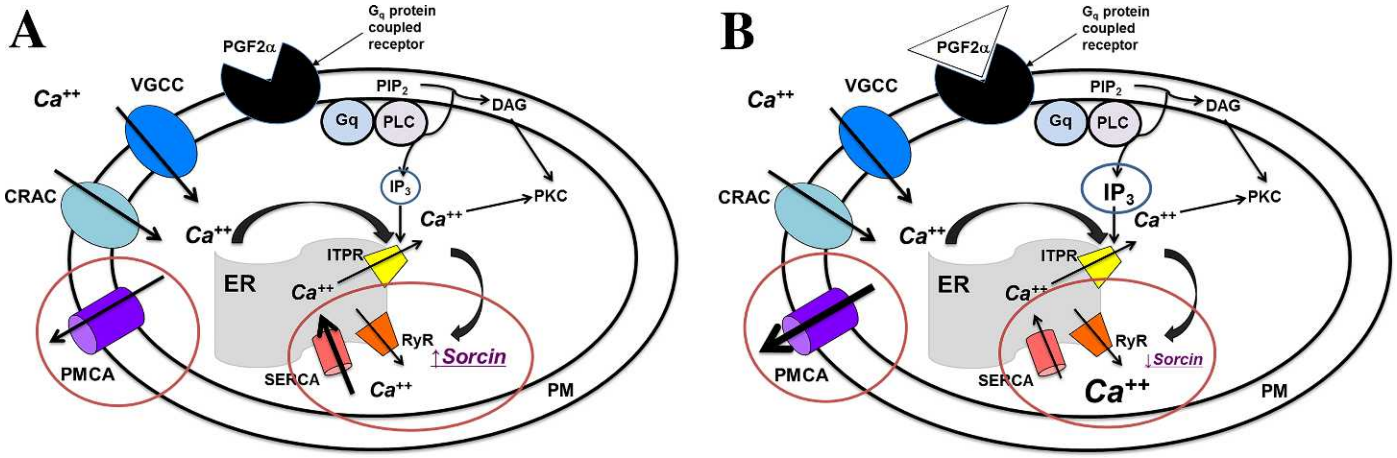


FIG. 10. Proposed model of the mechanisms of calcium homeostasis during luteal development. **A**) Developing CL: decreased phospholipase beta1 (PLC) activity; lower functional status of ryanodine receptor (RYR) due to increased expression of sorcin (SRI), which prevents RYR from opening; and greater expression of calcium transporting, cardiac, slow twitch 2 (ATP2A2 or SERCA). **B**) Mature CL: greater activity of PLC with an increase in inositol 1,4,5-trisphosphate (IP3), greater Ca^{2+} release from the ER via decreased SRI expression with full functional status of RYR, and reduction in calcium reuptake to the ER due to lower expression of SERCA. Ca^{++} (calcium ions), CRAC/Orai (calcium release-activated calcium modulator), DAG (diacylglycerol), ER (endoplasmic reticulum), PIP_2 (phosphatidylinositol 4,5-bisphosphate), ITPR (IP3 receptors), PKC (protein kinase C), PM (plasma membrane), PMCA (or ATP2B2 = ATPase, calcium transporting plasma membrane 2), VGCC (family of voltage-gated calcium channel).

developmental differences in calcium homeostasis may contribute to a difference in responsiveness to PGF2α.

In the plasma membrane, the only Ca^{2+}-ATPase pump whose expression increased significantly from developing to mature CL was the ATP2B1. These pumps transfer one Ca^{2+} to the outside of the cell per one ATP molecule hydrolyzed, and one H^+ is pumped inside of the cell [57]. This result may reflect that although mature CL have acquired the cellular basis for physiological sensitivity to luteolytic actions of PGF2α, the ability is retained to extrude enough cytoplasmic calcium to maintain $[Ca^{2+}]_i$ that are permissive to progesterone biosynthesis, which is normally high at this stage of luteal development. Although protein expression for ATP2B1 was examined using a commercial primary antibody, ab2825, we were unable to detect species cross immunoreactivity in bovine luteal tissue.

Both L and N types of VGCCs were expressed, but a developmental difference was not detected in bovine CL. Based on mRNA alone, it does not appear as if differences in expression of VGCCs mRNA could explain the developmental difference in calcium response to exogenous PGF2α. However, as with the ITPRs, these channels are regulated by phosphorylation and Ca^{2+} binding as well as proximal location to ER channels [58]. Therefore, their contribution to the developmental difference in response to PGF2α should not be disregarded. Pharmacological studies demonstrated that transport of Ca^{2+} via VGCCs participated in the luteinization process of porcine granulosal cells [59]. Expression and functional evidence of VGCCs in human CL (unknown luteal phase) and human luteinized granulosal cells have been reported, with both L and T types expressed and shown to be involved in steroidogenesis [35]. The difference in the type of these channels expressed in the human and cow may be species related.

Store-operated calcium channels are essential for $[Ca^{2+}]_i$ maintenance in response to intracellular calcium store depletion, a mechanism called store-operated Ca^{2+} entry [60]. Associated with this mechanism is a Ca^{2+}-release activated Ca^{2+} current (CRAC) that is activated on detecting a decrease in the amount of Ca^{2+} in the ER but not a rise in cytosolic $[Ca^{2+}]$ [61]. Although the exact mechanism is poorly understood, two components are known to be involved in store-operated Ca^{2+} entry: stromal-interaction molecule (STIM) and Orai. STIM, a calcium sensor protein, is localized on the ER membrane and plasma membrane [62, 63], and Orai, found on the plasma membrane, forms the pore of the store-operated calcium channel [64–66]. When the ER stores are low, STIM aids in the activation of Orai channels to permit extracellular Ca^{2+} to enter the cytosol [62, 67]. The ORAI primers used in this study were based on the human genes [68], which have high homology with the cow [69]. In this investigation, ORAI2 isotype was expressed in the bovine CL but was not developmentally regulated. However, the clustering of ORAI2 channels with the ER STIM proteins might be one of the factors necessary for regulation of this channel [67].

Single-cell experiments in which the effect of eliminating extracellular calcium in the presence or absence of a ryanodine on the PGF2α-stimulated rise in $[Ca^{2+}]_i$ was examined provided strong functional support for the interpretation that differential expression of RYR2 is likely to be part of the cellular basis responsible for the luteal developmental difference in calcium response to exogenous PGF2α. Removal of extracellular calcium reduced the calcium response elicited by PGF2α only in cells isolated from developing CL (Fig. 5). This indicates that in mature CL, the effect of PGF2α could be accounted for by calcium release from the ER. In contrast, in developing CL, calcium release was only a minor component, and extracellular calcium influx was more important in explaining the rise in $[Ca^{2+}]_i$ during PGF2α stimulation. Similarly, an effect of ryanodine in reducing the response elicited by PGF2α on increasing $[Ca^{2+}]_i$ could be demonstrated only in cells isolated from mature CL. Again, this indicates that in cells isolated from mature CL, the rise in $[Ca^{2+}]_i$ elicited by PGF2α stimulation had a major component that depended on calcium release from the ER, whereas mechanisms of calcium influx were more important in cells isolated from developing CL.

These observations might explain, at least in part, the reported developmental difference regarding the greater potency of PGF2α to induce an increase in $[Ca^{2+}]_i$ in the mature CL [19]. Choudhary et al. [19] demonstrated that

although luteal endothelial cells responded to PGF2α with an increase in $[Ca^{2+}]_i$, this response did not differ as a function of luteal development. Therefore, steroidogenic cells are the luteal population that contributes to developmental differences in gene/protein expression and PLCβ enzyme activity observed in the present study.

The importance of intracellular calcium in luteal steroidogenesis has been documented in several species, including the primate [27], mouse [30], sheep [21], and cow [17]. Additional studies are needed to fully comprehend the nature of the calcium regulatory mechanism in the bovine CL. Results of this study delineate the specific components and potential developmentally regulated steps of this complex aspect of luteal function and support the hypothesis that developmental differences in components involved in calcium homeostasis contribute, in part, to the ability of PGF2α to induce a rise in $[Ca^{2+}]_i$ of greater magnitude in mature than in developing bovine CL.

In summary, this study provides information regarding the specific cellular components of calcium homeostasis in the bovine CL. The results of this investigation constitute an initial necessary step in understanding the cellular mechanisms that might be at play during the events triggered by PGF2α in bovine CL at different developmental stages. The summarized results are depicted in a proposed model (Fig. 10). In the developing CL, the combined effects of 1) lower concentration of IP3 due to reduced PLCβ activity, 2) increased expression of SRI to prevent RYR2 from opening, and 3) greater expression of the component in calcium reuptake, ATP2A2, could account for the developmental difference in the ability of PGF2α to increase $[Ca^{2+}]_i$ to the degree observed in mature CL (Fig. 10A). It follows that in mature CL, the cellular mechanisms that allow PGF2α to induce a calcium signal of greater magnitude are the combined contribution of 1) an increase in IP3 due to a greater activity of PLCβ, 2) a greater Ca^{2+} release from the ER via increased functional opening of RYR2 due to a decrease in SRI expression, and 3) a reduction in calcium reuptake to the ER due to lower expression of ATP2A2 (Fig. 10B). These findings could be significant physiologically, as it was demonstrated previously that the ability of PGF2α to induce a decrease in LH-stimulated progesterone in mature bovine CL was associated closely with its ability to induce an increase in $[Ca^{2+}]_i$ [25]. The gene/protein expression data support the observation that in the mature bovine CL, the ER is the main source of the PGF2α-stimulated rise in $[Ca^{2+}]_i$. Greater expression of the plasma membrane calcium pump, ATP2B1, may allow the mature CL to maintain $[Ca^{2+}]_i$ to quantities that allow progesterone biosynthesis typical of the mid-luteal phase. Overall, the evidence in this study advances our understanding of mechanisms regulating calcium signaling in bovine CL and provides an additional potential explanation for insensitivity of the developing CL to PGF2α.

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