Control of ascorbic acid efflux in rat luteal cells: role of intracellular calcium and oxygen radicals

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Pepperell, John R., D. Marshall Porterfield, David L. Keefe, Harold R. Behrman, and Peter J. S. Smith. Control of ascorbic acid efflux in rat luteal cells: role of intracellular calcium and oxygen radicals. Am J Physiol Cell Physiol 285: C642–C651, 2003. First published April 30, 2002; 10.1152/ajpcell.00587.2002.—In luteal cells, prostaglandin (PG)F2α mobilizes intracellular calcium concentration ([Ca]i), generates reactive oxygen species (ROS), depletes ascorbic acid (AA) levels, inhibits steroidogenesis, and ultimately induces cell death. We investigated the hypothesis that [Ca]i mobilization stimulates ROS, which results in depletion of cellular AA in rat luteal cells. We used a self-referencing AA-selective electrode that noninvasively measures AA flux at the extended boundary layer of single cells and fluorescence microscopy with fura 2 and dichlorofluorescein diacetate (DCF-DA) to measure [Ca]i and ROS, respectively. Menadione, a generator of intracellular superoxide radical (O2·−), PGF2α, and calcium ionophore were shown to increase [Ca]i and stimulate intracellular ROS. With calcium ionophore and PGF2α, but not menadione, the generation of ROS was dependent on extracellular calcium influx. In unstimulated cells there was a net efflux of AA of 121.5 ± 20.3 fmol·cm−1·s−1 (mean ± SE, n = 8), but in the absence of extracellular calcium the efflux was significantly reduced (10.3 ± 4.9 fmol·cm−1·s−1; n = 5, P < 0.05). PGF2α and menadione stimulated AA efflux, but calcium ionophore had no significant effect. These data suggest two AA regulatory mechanisms: Under basal conditions, AA efflux is calcium dependent and may represent recycling and maintenance of an antioxidant AA gradient at the plasma membrane. Under luteolytic hormone and/or oxidative stress, AA efflux is stimulated that is independent of extracellular calcium influx or generation of ROS. Although site-specific mobilization of calcium pools and ROS cannot be ruled out, the release of AA by PGF2α-stimulated luteal cells may occur through other signaling pathways.

The corpus luteum of the mammalian ovary is a unique endocrine organ: hormones regulate not only its function but also its existence. During hormone-initiated luteolysis, the corpus luteum is selectively destroyed within the complex interrelationship of somatic, endocrine, and germ cells of the ovary. The major hormonal initiator of luteolysis is prostaglandin (PG)F2α. An initial event of luteolysis is loss of steroidogenic activity, and it is completed by the structural and cellular destruction of the corpus luteum by a process that includes apoptosis (16, 17, 41, 43). However, the mechanisms that bring about the specific cell-directed loss of function and ultimate cell death are incompletely understood. Because antioxidants, including ascorbic acid (AA), can suppress apoptosis in luteal cells (9, 58), the antioxidant status of the luteal cell may be key in the onset of luteolysis.

Cellular ascorbate regulation involves several transporter mechanisms. Cell membrane transporters including the sodium-dependent AA transporters SVCT2 and SVCT1 (56) directly transport AA. In addition, the glucose transporters GLUT-1 and GLUT-3 also play a role through transport of dehydroascorbic acid (DHA) (13, 44). DHA is a product that is formed when AA donates two electrons in its role as an antioxidant. DHA formed extracellularly, or released out of the intracellular environment, may be transported back into the cell via GLUT-1 and GLUT-3, where intracellular reduction of DHA regenerates AA (25, 26). These recycling pathways are important for primates, including humans, and other species that do not synthesize their own AA, and they provide a mechanism for maintenance of high intracellular levels of AA. Indeed, depending on the extracellular concentration of AA and DHA, some cells, including luteinized granulosal cells, could preferentially utilize the DHA pathway to maintain intracellular ascorbate levels, as indicated by the kinetics of uptake that show a 50-fold greater velocity for uptake for DHA compared with AA (18).

After exposure to PGF2α, luteal cells rapidly (within minutes) display mobilization of intracellular calcium concentration ([Ca]i), stimulation of calcium influx from the extracellular milieu (36), and generation of reactive oxygen species (ROS) (3, 42, 46). Furthermore, luteolysis that occurs spontaneously, and after PGF2α administration in vivo, is marked by a rapid depletion of the high concentration of AA that is normally

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present in the active corpus luteum (10, 38, 53). In vitro studies show that PGF₂α treatment of luteal cells inhibits uptake and stimulates release of radiolabeled AA (30). However, it is unclear whether ascorbate depletion occurs because of radical generation or whether ascorbate depletion promotes radical generation due to depleted cellular antioxidants.

It is our hypothesis that luteolytic signaling includes mobilization of \([Ca]_i\) that stimulates the formation of ROS, and that release of AA is a consequence of ROS formation in luteal cells. We have developed a noninvasive method for measuring the levels of AA near the extracellular face of the cell membrane and can detect its flux across the membrane in real time and in response to acute stimulation. Therefore, in the present study, we investigated the interrelationship of calcium mobilization, ROS formation, and AA flux in luteal cells in the early events of luteolytic stimulation.

METHODS

**Animals.** Immature (26–27 days old) female rats (CD strain; Charles River Laboratories, Wilmington, MA) were injected subcutaneously with 50 IU of pregnant mare serum gonadotrophin (PMSG, Gestyl; Organon Pharmaceuticals, West Orange, NJ) to induce superovulation. Fifty-four hours later, 25 IU of human chorionic gonadotrophin (hCG) (Wyeth-Ayerst Laboratories, Rouses Point, NY) was injected to induce ovulation and pseudopregnancy in accordance with institutional animal care and use committee guidelines.

**Luteal cell isolation.** Rat luteal cells were isolated by methods described previously (54). The ovaries were removed at day 5 to day 7 after hCG and minced with a razor blade. Tissue was digested in Ca²⁺-free minimal essential medium (medium 1; no. 1380, GIBCO, Grand Island, NY) containing 1% fetal calf serum (FCS; GIBCO) and 2,000 IU of collagenase ( Worthington Biochemical, Freehold, NJ) plus 3,000 IU of DNase ( Worthington) per gram of tissue for 1 h at 37°C under 95% air-5% CO₂.

The isolated luteal cells were enriched by density gradient sedimentation on a discontinuous density gradient (Percoll; New Jersey; Organon, Netherlands) containing 10% FCS, 100 U/ml penicillin, 0.1 μg/ml streptomycin, and 0.25 μg/ml amphotericin-B (all GIBCO). Cell incubations were accomplished by controlling the translational movement of a compound, if \(D\) is the distance between measurement positions (cm). Because \(D\) is given for a specific compound, if \(\Delta C/\Delta r\) can be measured continuously, real-time measurement of flux around a cell may be obtained. This is accomplished by controlling the translational movement of a selective electrochemical microelectrode through the gradient within the unstirred layer next to the membrane. The electrode tip is moved through the extracellular gradient at a known frequency and between known points. By signal averaging the electrode output obtained at each position and then referencing the signals together, a differential signal is obtained that can be converted into a directional measurement of flux with the Fick equation. Referencing the signals in this manner confers the advantage that sources of interference caused by random drift and noise are effectively filtered from the signal (19, 51). This technique belongs to a family of analytical approaches incorporating modulation techniques. In this application, it is termed self-referencing.

**AA flux measurement.** The basic concepts of the self-referencing microelectrode have been described in detail previously (19, 49–51) but were modified for application with an electrochemical AA microprobe. Like the ion probe application that we have previously used (35, 52), measurement of electrochemically active compounds fluxing from single cells is based on the description of the diffusionary movement of ions and gases in solution by the Fick equation

\[
J = -D \frac{\Delta C}{\Delta r} \tag{1}
\]

where \(J\) is the flux (mol·cm⁻²·s⁻¹), \(D\) is the diffusion coefficient for AA (6.6 \times 10⁻⁶ cm/s), \(\Delta C\) is the concentration difference (mol/ml), and \(\Delta r\) is the distance between measurement positions (cm). Because \(D\) is given for a specific compound, if \(\Delta C/\Delta r\) can be measured continuously, real-time measurement of flux around a cell may be obtained. This is accomplished by controlling the translational movement of a selective electrochemical microelectrode through the gradient within the unstirred layer next to the membrane. The electrode tip is moved through the extracellular gradient at a known frequency and between known points. By signal averaging the electrode output obtained at each position and then referencing the signals together, a differential signal is obtained that can be converted into a directional measurement of flux with the Fick equation. Referencing the signals in this manner confers the advantage that sources of interference caused by random drift and noise are effectively filtered from the signal (19, 51). This technique belongs to a family of analytical approaches incorporating modulation techniques. In this application, it is termed self-referencing.

**Intracellular calcium concentration measurements.** [Ca]ₗ in rat luteal cells was determined with fura 2 by established methods (36, 40). Emission of fura 2 saturated with calcium and in the absence of calcium was obtained in each experiment by the addition of ionomycin (10 μM) and EGTA (10 mM), respectively, to the cells.
onal array and driven by size 23 stepper motors. This arrangement provided nano- to millimeter resolution of movement of the head stage in either an oscillating (square wave at 0.3 Hz) or static mode. Control over movement was achieved by computer control with IonView software developed at the BioCurrents Research Center (BRC). The entire assembly was mounted on an antivibration table and housed within a Faraday cage equipped with a temperature control system. Data were collected by using IonView software (BRC) at a rate of 1,000 data points/s and signal averaged into 10 values consisting of 166 data points each. Values obtained over the first 3/10ths of translation, including the period of electrode movement, were discarded.

Flux calculations. Electrochemical sensors characteristically respond linearly to substrate concentration and the differential current output is

\[ C_1 - C_2 = (sI_1 + b) - (sI_2 + b) \]  

where \( I \) is the electrode output (\( \Delta I \)), \( C \) is the concentration (\( \text{mmol/ml} \)), and \( s \) and \( b \) are the slope and \( y \)-intercept of the linear calibration, respectively. This equation can be rearranged to the form

\[ \Delta C = s\Delta I \]  

\( \Delta C \) can now be reconciled with the amplitude of electrode oscillation (\( \Delta C \)), which in these experiments was 30 \( \mu \text{m} \), to obtain a measurement of flux (expressed as \( \text{mmol/cm}^2 \cdot \text{s}^{-1} \)) with the Fick equation (Eq. 1).

\[ J = -D \frac{s\Delta I}{\Delta C} \]  

AA microsensors. For the development of a self-referencing AA system, we used an electrochemically modified carbon disk electrode. The basic electrode was constructed by modifying and combining two previously described protocols (4, 11). Briefly, this involved pulling a 5-\( \mu \text{m} \) carbon fiber (Amoco, Greenville, SC) in a glass microcapillary. When the glass was heated and pulled, it was drawn down to make contact with the fiber. The fiber was stabilized and sealed in the pulled glass pipette with epoxyite (Epoxyite, Westerville, OH). After the epoxyite was allowed to cure in an oven at 110°C for 5–10 h, the electrode was backfilled with a graphite-epoxy paste (PX-grade Graphpoxy; Dylon Industries, Cleveland, OH). A copper wire was then inserted, making electrical contact with the carbon fiber through the graphite-epoxy paste. The paste was cured (110°C for 5–10 h), and the excess carbon fiber was chopped with a scalpel and beveled to 30°. These electrodes were then electrochemically treated according to previous studies (4, 14, 15). A 70-Hz triangle wave that cycled between +3.0 and +1.5 V (7 s) was followed by a constant potential of +1.5 V (7 s) and then held at 0.0 V (30 min). The electrochemical treatment process decreases the polarization voltage required for oxidation of AA down to the 0.15- to 0.3-V range. The voltage applied in these studies was 0.3 V, which, as described in DISCUSSION, gives good selectivity for AA over potentially interfering compounds.

AA flux measurements in luteal cells. Rat luteal cells plated on glass coverslips that replaced the bottom of a 2-ml culture dish were cultured in DMEM supplemented with 10% FCS as described in Luteal cell isolation. Flux experiments were conducted after culture for 24–48 h, when medium was removed and replaced with DMEM supplemented with a system to generate DHA in situ that consisted of AA (300 \( \mu \text{M} \)) plus ascorbic acid oxidase (2 U/ml) and thiourea (500 \( \mu \text{M} \)). This system is routinely used to add DHA to cells, because it completely oxidizes AA to DHA within 10 min and overcomes the impurity of commercially available DHA (18). Luteal cells were incubated for 60 min before being washed five times with DPBS. The cells were incubated for 10 min at 37°C and then washed three times with DPBS before being placed on the stage of the microscope attached to the electrophysiology apparatus that was maintained at 37°C within the Faraday cage. (Thermal control was achieved by heating the air table and insulated box, microscope, and manipulators; BRC). A charge-coupled device (CCD) camera imaged cells, and the carbon fiber electrode was brought close (within 1 \( \mu \text{m} \)) to the cell membrane of a plated luteal cell with the orthogonal array of stepper motors of the electrode rig.

Statistical analysis. Data are presented as means ± SE. Statistical differences between two means were assessed by Student's t-test, whereas differences between multiple means were determined by one-way analysis of variance (ANOVA) followed by Neuman-Keuls test for significance or Kruskal-Wallis one-way ANOVA followed by Dunn's method for determining significance between groups. A significant difference was established at the level of \( P < 0.05 \). Each experiment was replicated a minimum of three times with luteal cells prepared on at least two different days.

Hormones, drugs, and reagents. PGF2α was purchased from Upjohn (Kalamazoo, MI). Fura 2-AM and DCF-DA were purchased from Molecular Probes (Eugene, OR) and were dissolved in dry dimethyl sulfoxide (DMSO) immediately before use. The final concentration of DMSO in the incubation medium was not greater than 0.7%. Menadione was obtained from Sigma (St. Louis, MO). Ionomycin was obtained from Calbiochem (San Diego, CA). EGTA was obtained from Fluka Chemical (Ronkonkoma, NY).

RESULTS

ROS generation and steroidogenesis. Menadione is an intracellular generator of superoxide radical (O2-) in mitochondria (23), and because the natural luteolysin PGF2α is also known to stimulate luteal ROS production, we examined the effects of menadione on steroidogenesis in isolated luteal cells. Figure 1 shows that menadione dose-dependently inhibits both basal and LH-stimulated progesterone production by freshly isolated luteal cells, with a significant (\( P < 0.05 \)) degree of inhibition achieved with a 5 \( \mu \text{M} \) dose.

Menadione and [Ca2+]i mobilization. PGF2α is antigonadotropic, generates ROS, and mobilizes [Ca2+]i. Therefore, we investigated whether menadione also mobilizes [Ca2+]i in luteal cells. At a concentration of 5 \( \mu \text{M} \) menadione induced a small increase in [Ca2+]i, but at higher concentrations there was a sustained elevation (Fig. 2). The role of extracellular calcium in [Ca2+]i mobilization induced by menadione was assessed in calcium-free DPBS supplemented with EGTA (0.5 mM). Table 1 summarizes the data and shows that in calcium-replete medium menadione (50 \( \mu \text{M} \)) elevated basal [Ca2+]i from 89 ± 11 nM to a peak level of 265 ± 39 nM, which was sustained for at least 500 s (204 ± 20 nM). In the absence of extracellular calcium menadione (50 \( \mu \text{M} \)) increased [Ca2+]i from a basal level of 62 ± 5 nM to a level of 98 ± 11 nM (\( P < 0.05 \)) that was also sustained for at least 500 s, but this level of increase
was significantly smaller than that observed in the presence of extracellular calcium ($P < 0.001$).

To further compare the effects of menadione and PGF$_{2\alpha}$, we examined whether menadione depletes intracellular calcium pools. PGF$_{2\alpha}$ was added to cells after treatment with menadione (50 $\mu$M) and was found to induce a further increase in $[Ca]_i$, in both the presence and the absence of extracellular calcium (Table 2), indicating that intracellular stores were not totally depleted or immobilized by menadione.

**ROS generation in luteal cells.** To validate the measurement of ROS generation in luteal cells, we added H$_2$O$_2$ to DCF-DA-loaded cells and measured the increase in fluorescence of dichloro fluorescein (DCF) during excitation from 425 to 500 nm. The fluorescence recorded during excitation at 450 nm was less sensitive to H$_2$O$_2$ than the fluorescence recorded during excitation at 500 nm, and when the fluorescence ratio (500/450 nm) was expressed as a rate increase over 200 s, a linear relationship with H$_2$O$_2$ concentration was evident (Fig. 3). We next examined the effects of PGF$_{2\alpha}$, calcium ionophore, and menadione on luteal ROS production. PGF$_{2\alpha}$ was used at a concentration that is maximally effective for inhibition of steroidogenesis (36, 54), and calcium ionophore was used at a concentration of 1 $\mu$M, which has antigonadotrophic effects and mobilization of $[Ca]_i$ similar to that of PGF$_{2\alpha}$ (36). Table 3 summarizes the ROS generation stimulated by PGF$_{2\alpha}$, ionomycin, and menadione over the initial 200 s of treatment. Addition of buffer alone did not significantly change the fluorescence of DCF, but PGF$_{2\alpha}$, ionomycin, and menadione all significantly increased fluorescence over the initial basal levels ($P < 0.05$). ANOVA detected no significant difference between the increase in fluorescence induced by PGF$_{2\alpha}$, ionomycin, or 25 $\mu$M menadione ($P > 0.05$); however, the increase in ROS induced by a higher concentration of menadione (50 $\mu$M) was statistically significant ($P < 0.05$). When experiments were repeated with medium that contained zero calcium and was supplemented with EGTA (0.5 mM), no significant effect of either PGF$_{2\alpha}$ or calcium ionophore was detected on DCF fluorescence (Table 4).

Table 1. Role of extracellular calcium in intracellular calcium mobilization induced by menadione

<table>
<thead>
<tr>
<th></th>
<th>$n$</th>
<th>Basal</th>
<th>Menadione-Induced Peak</th>
<th>500 s Post-treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca-replete medium</td>
<td>5</td>
<td>$89 \pm 11$</td>
<td>$265 \pm 39^a$</td>
<td>$204 \pm 20$</td>
</tr>
<tr>
<td>Ca-depleted medium</td>
<td>7</td>
<td>$62 \pm 5$</td>
<td>$98 \pm 11^a$</td>
<td>$96 \pm 8$</td>
</tr>
</tbody>
</table>

Data (in nM) are means $\pm$ SE of peak values for $n$ replications of intracellular calcium concentration ($[Ca]_i$) induced by menadione and values at 500 s after its addition. Luteal cell $[Ca]_i$ was determined in Dulbecco's phosphate-buffered saline (DPBS) containing 1.8 mM calcium (Ca-replete) and calcium-free DPBS supplemented with EGTA (1 mM) (Ca-depleted). Menadione was added at a final concentration of 50 $\mu$M.

**Fig. 1.** Effect of menadione on progesterone production. Luteal cells were incubated for 1 h in the absence or presence of LH (100 ng/ml) and increasing concentrations of menadione. Progesterone was measured in cells and medium. Data represent means $\pm$ SE of triplicate replications. *Significantly different from progesterone in the absence of menadione ($P < 0.05$).

**Fig. 2.** Effects of menadione on intracellular Ca concentration ($[Ca]_i$). Representative traces from experiments that were replicated a minimum of 3 times are shown.
AA flux measurements. We tested and validated the measurement of AA flux against theoretical values in a gradient created with an AA source. For this, an AA source pipette with a diameter of 10 μm was prepared with 0.66 M ascorbate in a 0.33% agar matrix. The gradient was characterized and modeled so that measured flux values could be compared with the theoretically derived values by using the model described in Land et al. (20). The relationship between measured values compared favorably with the theoretically derived values (Fig. 4).

AA flux in luteal cells was initially measured under basal conditions in the presence or absence of extracellular calcium. Data were collected continuously over a 5-min period, and Table 5 shows the mean flux obtained. In the presence of extracellular calcium, a net efflux of AA was recorded at the luteal cell membrane (121.5 ± 20.3 fmol·cm⁻¹·s⁻¹) that was significantly different (P < 0.001) from that recorded at a background distance of 500 μm from the cells (−12.6 ± 7.2 fmol·cm⁻¹·s⁻¹). When the medium contained zero calcium and was supplemented with EGTA (0.5 mM), AA flux was markedly attenuated and was significantly less (P < 0.001) than that observed in the presence of calcium.

We next tested the effects of calcium ionophore and PGF₂α on ascorbic acid flux. In Table 6 flux was measured continuously for 5 min before and after the addition of agent and is expressed as the mean ± SE of replicated experiments, whereas Fig. 5 describes the real-time pattern of flux. PGF₂α (1 μM) induced a rapid and significant stimulation of efflux (P < 0.05) that peaked immediately after treatment but maintained sustained efflux for up to 10 min. In contrast, a slight increase in efflux after calcium ionophore (1 μM) was not a statistically significant effect (P > 0.05). In some experiments extracellular calcium was removed (calcium-free DPBS supplemented with 0.5 mM EGTA), but this did not prevent PGF₂α-dependent stimulation of AA efflux (data not shown).

To further investigate AA flux, we tested the effects of menadione (50 μM) and found an immediate, large efflux of AA that was sustained for at least 20 min (Fig. 6). However, pretreatment of luteal cells with PGF₂α or ionomycin modified the menadione response (Fig. 7). Menadione (50 μM) induced an increase in net efflux in both groups; however, the efflux in cells that had been pretreated with ionomycin (1 μM) was significantly different (P < 0.001) from that of cells pretreated with PGF₂α (1 μM) and was sustained.

Table 2. Effect of PGF₂α on [Ca]i

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Peak [Ca]i</th>
<th>∆[Ca]i</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca-replete medium</td>
<td>5</td>
<td>250 ± 25</td>
</tr>
<tr>
<td>Ca-depleted medium</td>
<td>7</td>
<td>150 ± 15</td>
</tr>
</tbody>
</table>

Values are means ± SE of n replicates and show the peak [Ca]i and the change in [Ca]i (∆[Ca]i) induced by prostaglandin (PGF)₂α. [Ca]i was determined in luteal cells incubated in calcium replete and calcium-depleted DPBS as in Table 1. Cells were pretreated with calcium-depleted DPBS as in Table 1. Cells were pretreated with calcium-replete medium 5 250 44 19

Table 3. Stimulation of ROS generation by PGF₂α, calcium ionophore, and menadione

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>ROS Generation, Δ500/450-nm fluorescence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>11</td>
<td>2.29 ± 1.96 x 10⁻⁵</td>
</tr>
<tr>
<td>PGF₂α (1 μM)</td>
<td>8</td>
<td>14.16 ± 3.81 x 10⁻⁵</td>
</tr>
<tr>
<td>Calcium ionophore (1 μM)</td>
<td>6</td>
<td>9.45 ± 2.34 x 10⁻⁵</td>
</tr>
<tr>
<td>Menadione</td>
<td>5</td>
<td>14.45 ± 3.58 x 10⁻⁵</td>
</tr>
<tr>
<td>25 μM</td>
<td>7</td>
<td>72.56 ± 2.55 x 10⁻⁵</td>
</tr>
<tr>
<td>50 μM</td>
<td>7</td>
<td>72.56 ± 2.55 x 10⁻⁵</td>
</tr>
</tbody>
</table>

Values are means ± SE of n determinations. Acute formation of intracellular reactive oxygen species (ROS) was assayed by dichlorofluorescein diacetate (DCFDA) in luteal cells by measuring the increase in the ratio of fluorescence during 500/450 nm excitation as described in Fig. 3. *Significant effect of treatment on the rate of fluorescence increase. Means with different superscript letter are significantly different from each other (P < 0.05).

Table 4. Effect of PGF₂α and calcium ionophore on ROS generation in calcium-free medium

<table>
<thead>
<tr>
<th>Treatment in Ca²⁺-Chelated Medium</th>
<th>n</th>
<th>ROS Generation, Δ500/450-nm fluorescence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>8</td>
<td>0.31 ± 1.42 x 10⁻⁵</td>
</tr>
<tr>
<td>PGF₂α</td>
<td>3</td>
<td>4.21 ± 2.80 x 10⁻⁵</td>
</tr>
<tr>
<td>Calcium ionophore</td>
<td>4</td>
<td>5.21 ± 2.12 x 10⁻⁵</td>
</tr>
</tbody>
</table>

Values are means ± SE for n replicates. The effects of PGF₂α and ionophore on DCFDA-loaded luteal cells incubated in calcium-free medium supplemented with EGTA (0.5 mM) were determined as described in Table 3. No significant effects on fluorescence were detected.

Fig. 3. Determination of intracellular reactive oxygen species (ROS) generation. Effect of increasing concentration of H₂O₂ on the rate of increase in fluorescence ratio (500/450 nm). Fluorescence emission at 520 nm was simultaneously recorded during dual excitation at 450 and 500 nm. The increase in fluorescence measured over 200-s exposure to H₂O₂ was computed as a rate increase in fluorescence ratio and plotted against peroxide concentration. Inset: wavelength scan of dichlorofluorescein (DCF) diacetate (DCF-DA)-loaded luteal cells in the presence or absence of H₂O₂.
DISCUSSION

Early events of luteolytic signaling include mobilization of intracellular calcium (8, 36), generation of O$_2$ (3, 61, 62) and H$_2$O$_2$ (42), and formation of lipid peroxides (2, 46), but it is unclear whether radical formation is a consequence of calcium mobilization or a parallel event in early luteolysis. In the present study we investigated [Ca]$^2+$ mobilization, generation of ROS, and flux of AA during luteolytic stimulation to determine the sequence of these events.

PGF$_{2\alpha}$ is a natural luteolysin, and it elevates [Ca]$^2+$ in luteal cells, mainly from mobilized endoplasmic reticulum stores and extracellular influx (8, 36). On the other hand, calcium ionophore releases calcium from all internal stores including mitochondria, as well as stimulating extracellular influx. In this study, menadione induced a rapid, large increase in [Ca]$^2+$ and removal of extracellular calcium abrogated the increase in [Ca]$^2+$ with little or no effect on subsequent calcium release by PGF$_{2\alpha}$. This suggests that menadione principally induces calcium influx from extracellular media with little or no effect on the endoplasmic stores. The influx may derive from menadione-induced generation of ROS in mitochondria (55) that can cause dysfunction of the organelle and impaired ATP generation. The absence of energy-dependent ion regulation permits calcium to flow down the concentration gradient and influx from extracellular sources. The inhibitory effect of menadione on steroidogenesis may also be directly attributable to impaired mitochondrial function. The small increase in [Ca]$^2+$ induced by menadione when extracellular calcium was chelated may be due to release from mitochondrial stores, as that organelle's ATP levels are depleted.

Production of ROS during the early events of luteolysis is implicated in several species, (9, 42, 58), but

Table 5. Effect of calcium on ascorbic acid flux in luteal cells

<table>
<thead>
<tr>
<th></th>
<th>Ca Replete ($n = 8$)</th>
<th>Ca Free ($n = 5$)</th>
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<tbody>
<tr>
<td>Mean ± SE</td>
<td>121.5 ± 20.3$^{a,a}$</td>
<td>10.3 ± 4.9$^{b,b}$</td>
</tr>
<tr>
<td>Background</td>
<td>−12.6 ± 7.2</td>
<td>−14.4 ± 3.6</td>
</tr>
</tbody>
</table>

Data were recorded continuously over 5 min, and all values (in fmol·cm$^{-2}$·s$^{-1}$) were used for statistical analysis; $n$ represents the number of replications for each group. Ascorbic acid flux was measured with an ascorbic acid-sensitive electrode. Flux was recorded at the cell membrane and at a distance of 500 μm from the cell (background) in calcium-replete or calcium-free DPBS. Significantly different from background values ($P < 0.001$). Means with different superscript letters are significantly different from each other ($P < 0.001$). Positive flux values indicate efflux, and negative flux values indicate influx of ascorbic acid.

Fig. 5. Real-time ascorbic acid flux in luteal cells and the effects of prostaglandin (PGF$_{2\alpha}$) and calcium ionophore. In individual experiments, continuously accumulated data were averaged at 30-s intervals and the mean value from the initial 5-min period was used to compute the basal flux. Changes in flux expressed as a percentage of basal flux are shown, and each point represents the mean ± SE of $n$ replicated experiments: PGF$_{2\alpha}$, $n = 4$; ionophore, $n = 7$. PGF$_{2\alpha}$ vs. basal, $P < 0.05$.

Table 6. Effects of PGF$_{2\alpha}$ and calcium ionophore on ascorbic acid flux

<table>
<thead>
<tr>
<th></th>
<th>PGF$_{2\alpha}$ ($n = 8$)</th>
<th>Calcium Ionophore ($n = 6$)</th>
</tr>
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<tbody>
<tr>
<td>Basal</td>
<td>96.7 ± 12.8</td>
<td>55.9 ± 6.5</td>
</tr>
<tr>
<td>Treatment</td>
<td>217.4 ± 42.2$^a$</td>
<td>65.5 ± 10.1</td>
</tr>
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Values (in fmol·cm$^{-2}$·s$^{-1}$) are means ± SE ascorbic acid flux for $n$ replications. Ascorbic acid flux was measured continuously for 5 min before the addition (basal) and after the addition (treatment) of PGF$_{2\alpha}$ or ionophore. All accumulated data were used to calculate the means ± SE. *Statistically significant difference from basal values ($P < 0.05$).
the mechanisms of their generation remain to be fully elucidated. Cytokines induce cell death by ROS-dependant mechanisms in luteal cells (39), and a protein kinase C (PKC)-dependent generator of O₂⁻ is present in cells of the corpus luteum (3, 45). This generator may be principally located in the cell population that is not LH sensitive (3). Furthermore, a direct effect of PGF₂α on O₂⁻ production in steroidogenic cells was not detected (3), but this may have been related to the use of luminol to detect the radical in cell suspensions. Therefore, the cell type and signal responsible for initial radical production in luteolysis require clarification. In the present study, we used plated luteal cells adhering to coverslips and DCF-DA, which measures intracellular generation of ROS. We found that for at least 3 min immediately after treatment with PGF₂α, DCF fluorescence increased, indicating generation of ROS. Previously we found (36) that PGF₂α induces [Ca]ᵢ mobilization within this time frame, and it will activate PKC in luteal cells (29) that may activate generators of ROS. DCF-loaded luteal cells also responded with increased fluorescence to ionomycin over a time frame similar to that of mobilized [Ca]ᵢ, and we previously found (36) that [Ca]ᵢ is elevated to similar levels by ionomycin and PGF₂α at the concentrations used here. However, in these studies when extracellular calcium was chelated there was no significant effect on DCF fluorescence by either PGF₂α or ionophore, suggesting that the major signal for ROS production is derived from calcium influx rather than release from intracellular stores.

To further investigate the role of ROS in luteolytic signaling we used menadione, which, after its one-electron reduction in mitochondria, produces a semiquinone radical that rapidly reduces dioxygen to form O₂⁻ (55, 60). It is a pharmacological tool used here to investigate radical-dependent calcium flux because it stoichiometrically produces O₂⁻ and may represent the physiological production of O₂⁻ that occurs during incomplete respiration and in aging mitochondria (18, 60). We found that menadione induced a rapid, dose-dependent increase in fluorescence in DCF-loaded cells that is consistent with O₂⁻ generation within the mitochondria.

The active corpus luteum maintains AA at high intracellular levels that are hormonally regulated (30, 32); however, these mechanisms are not fully understood. In the present study, we examined the acute regulation of AA flux at the luteal cell membrane in real time with a carbon fiber microelectrode. Previously, carbon fiber microelectrodes have been used with brain tissue in vivo to measure glutamate-induced release of AA by amperometry (4). Amperometric detection of redox compounds at the surface of the carbon fiber tip is a function of the redox potential of the compound, the oxidative state of the carbon surface, and the electropotential applied across the electrode surface. Selectivity for measurement of AA is achieved through electrochemical oxidation of the carbon fiber electrode by application of a cyclical triangular potential during its fabrication. This pretreatment results in the oxidation potential required to measure nitric oxide (NO) and catecholamines on carbon being much higher (in the 0.9-V range) compared with that needed for ascorbic acid (0.15–0.3 V) (4, 11, 33). Furthermore, the reduction-dependent peak potential for conversion of NO to N₂O occurs at a more negative potential than used in the present study (63). Thus, although other redox reactions may contribute some signal, these are relatively minor compared with the signal derived from oxidation of AA and the electrode is therefore selective for AA.

In its antioxidant role, AA can sequentially donate two electrons: the first donation yields the AA free radical (AFR), and the second yields DHA. Both AFR and DHA can be reduced back to AA, thereby recycling and conserving this important antioxidant. A major component of intracellular AA recycling is via glutathione.

![Graphic representation of the effect of menadione on ascorbic acid efflux.](image-url)

**Fig. 6.** Effect of menadione on ascorbic acid efflux. A representative experiment showing the effects of menadione on ascorbic acid efflux is displayed, and each point is the mean ± SE of this data accumulation. Flux was measured adjacent to a luteal cell and at a background distance of 500 μm from the cell.

![Graphic representation of the effect of PGF₂α and calcium ionophore ionomycin on menadione-induced ascorbic acid efflux.](image-url)

**Fig. 7.** Effect of PGF₂α and calcium ionophore ionomycin on menadione-induced ascorbic acid efflux. Data were continuously recorded and averaged at 30-s intervals. Each data point represents the mean ± SE of n replicated experiments: PGF₂α, n = 4; ionophore, n = 7. PGF₂α vs. ionophore, P < 0.001.
one (GSH)-dependent pathways (22, 28); however, extracellular mechanisms also contribute to recycling. For example, liver cells (31), K562 cells (47), and erythrocytes (27) recycle extracellular AA by electron transfer to AFR by presumed transmembrane activity of reductases that can utilize NADH or intracellular AA for reducing equivalents (31, 57). Coenzyme Q may also augment plasma membrane NADH-reductase that recycles AFR to AA and stabilizes the AA gradient at the membrane (1). In addition, extracellular reduction of DHA may also occur in some cell types (48). Maintenance of an AA gradient at the cell membrane may be key for its integrity and function.

The self-referencing microelectrode used in these studies detected changes in AA concentration within a discrete, micrometer-level region of the luteal cell membrane that were computed as AA flux. Under basal conditions, we measured a net efflux of AA at the cell membrane. This efflux may represent a low-level release of AA from intracellular stores. On the other hand, the possibility exists that under basal conditions we measured the activity of AFR or DHA recycling at the cell membrane to produce AA. The observation that this measurement of efflux was diminished in the presence of EGTA and zero extracellular calcium may indicate a dependence of the reductase activity on Ca$^{2+}$ or another divalent ion that is chelated by EGTA, such as Mg$^{2+}$. Extracellular AA may be transported back into the cell by SVCT2; however, luteinized cells also utilize DHA for maintenance of ascorbate levels (18). Thus oxidation of AA by other cells, or release of intracellular DHA, may be taken up by luteal cells on the GLUT-1 or -3 transporters (24, 26, 44).

AA efflux was stimulated by PGF$_{2\alpha}$, which is a finding consistent with studies that measured PGF$_{2\alpha}$-dependent release of radiolabeled AA from luteal cells (30). However, using the self-referencing electrode has enabled us to detect changes in AA flux during real time. The time frame of stimulated AA efflux was similar to that previously found for PGF$_{2\alpha}$-induced mobilization of [Ca]$^{2+}$ (36) and formation of intracellular ROS. However, it is not clear that calcium mobilization and formation of ROS is a regulator of stimulated ascorbate efflux: PGF$_{2\alpha}$ and calcium ionophore have similar effects on [Ca]$^{2+}$ and generation of ROS, but calcium ionophore had little or no effect on the acute efflux of AA. Previously in luteal cells, ionophore was shown to induce a small release from a pool of radiolabeled AA over a 30-min incubation. In comparison, the present study measured AA flux in real time and the results represent the net flux over 5–15 min, excluding the influence of individual ascorbate pools. Therefore, it seems possible that the apparent differences between the two studies are due to separate pools of AA and different durations of observation. The pool of AA released by PGF$_{2\alpha}$ is unclear. AA is a soluble antioxidant found in the cytoplasm, but it also accumulates in mitochondria (21) and the endoplasmic reticulum (7). In astrocytes activation of glutamate receptors mobilizes intracellular calcium stores and stimulates calcium influx (12, 34) and the production of ROS (6). Glutamate regulates anionic channels that release large amounts of AA and are responsible for the increase in extracellular AA in brain after trauma (59). It remains to be determined whether PGF$_{2\alpha}$ affects anionic channels in luteal cells that release AA, and whether calcium or ROS may regulate these potential channels directly, or whether some other signaling pathway initiates the release.

Menadione stimulated a massive net efflux of AA from luteal cells. The rate of efflux decreased with time, indicating reduced release, increased reuptake, or both. However, menadione induced a chronic net efflux, which is consistent with earlier studies that measured menadione-induced release of radiolabeled AA (29). Part of this release may be derived from mitochondrial stores of AA that are oxidized and released into the cytoplasm as AFR or DHA where recycling pathways are activated to form AA. Cell membrane channels or transporters may release the subsequent elevation in cytoplasmic ascorbate to the extracellular milieu. It cannot be ruled out that a component of efflux is the measurement of released AFR or DHA that is reduced and recycled to AA at the extracellular membrane. Menadione inhibits uptake of DHA, but it is not known whether AA uptake is affected (18, 30).

The response to menadione was also used to identify potential different effects of calcium ionophore and PGF$_{2\alpha}$ on AA regulation. Luteal cells pretreated with ionomycin exhibited a more sustained efflux of AA compared with luteal cells pretreated with PGF$_{2\alpha}$. Previously, uptake of radiolabeled AA was shown to be inhibited by PGF$_{2\alpha}$ but not ionomycin (30). Thus the dual effect of PGF$_{2\alpha}$ on stimulation of secretion and inhibition of uptake will have a net effect to deplete luteal AA. Our finding that AA efflux after menadione was more sustained in cells pretreated with ionomycin compared with cells pretreated with PGF$_{2\alpha}$ is consistent with depletion of luteal ascorbate in the latter group. In addition, the data are consistent with ionomycin having no effect on recycling pathways, which enabled intracellular ascorbate levels to be replenished and thus allowed a sustained efflux in response to menadione. These data indicate that menadione and the self-referencing electrode may be used to measure cellular AA content.

In the present study, we used a novel technique that noninvasively measures AA flux in cells and found under basal conditions a net efflux of AA that is affected by extracellular calcium. This efflux may represent a mechanism that recycles AA at the extracellular membrane and serves an antioxidant role at the plasma membrane. We also determined that AA efflux is stimulated by PGF$_{2\alpha}$ and menadione. Although both of these agents elevate [Ca]$^{2+}$, albeit by differing mechanisms, and generate intracellular ROS, it is not clear that these were signaling events for stimulation of AA efflux, because calcium ionophore that increases [Ca]$^{2+}$ levels and stimulates formation of luteal ROS had no significant effect on AA efflux. Although the importance of local elevation of [Ca]$^{2+}$ and site-specific gener-
atation of ROS are unknown, the data here indicate that luteolytic regulation of AA efflux is not dependent on calcium influx. Other mechanisms may drive PGF$_{2\alpha}$-dependent stimulation of AA efflux.

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DISCLOSURES

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