Subplasmalemmal hydrogen peroxide triggers calcium influx in gonadotropes

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Gonadotropin-releasing hormone (GnRH) stimulation of its eponymous receptor on the surface of endocrine anterior pituitary gonadotrope cells (gonadotropes) initiates multiple signaling cascades that culminate in the secretion of luteinizing and follicle-stimulating hormones, which have critical roles in fertility and reproduction. Enhanced luteinizing hormone biosynthesis, a necessary event for ovulation, requires a signaling pathway characterized by calcium influx through L-type calcium channels and subsequent activation of the mitogen-activated protein kinase extracellular signal-regulated kinase (ERK). We previously reported that highly localized subplasmalemmal calcium microdomains produced by L-type calcium channels (calcium sparklets) play an essential part in GnRH-dependent ERK activation. Similar to calcium, reactive oxygen species (ROS) are ubiquitous intracellular signaling molecules whose subcellular localization determines their specificity. To investigate the potential influence of oxidant signaling in gonadotropes, here we examined the impact of ROS generation on L-type calcium channel function. Total internal reflection fluorescence (TIRF) microscopy revealed that GnRH induces spatially restricted sites of ROS generation in gonadotrope-derived αT3-1 cells. Furthermore, GnRH-dependent stimulation of L-type calcium channels required intracellular hydrogen peroxide signaling in these cells and in primary mouse gonadotropes. NADPH oxidase and mitochondrial ROS generation were each necessary for GnRH-mediated stimulation of L-type calcium channels. Congruently, GnRH increased oxidation within subplasmalemmal mitochondria, and L-type calcium channel activity correlated strongly with the presence of adjacent mitochondria. Collectively, our results provide compelling evidence that NADPH oxidase activity and mitochondria-derived hydrogen peroxide signaling play a fundamental role in GnRH-dependent stimulation of L-type calcium channels in anterior pituitary gonadotropes.

The hypothalamic-pituitary-gonadal axis orchestrates reproductive function. Hypothalamic-pituitary-gonadal axis signaling begins with the release of gonadotropin-releasing hormone (GnRH),2 a hypothalamic neuropeptide, into the hypophysial portal system. Subsequent activation of GnRH receptors on anterior pituitary gonadotropes leads to increased synthesis and secretion of luteinizing hormone (LH) and follicle-stimulating hormone (FSH). In females, a dramatic gonadotrope GnRH receptor–dependent rise in LH is critical for ovulation and as such is a mandatory event for reproduction and fertility. Thus, the biological importance of molecular mechanisms regulating LH synthesis cannot be understated.

Activation of the GnRH receptor leads to canonical Gαq protein signaling where phospholipase C cleaves phosphatidylinositol 4–5-biphosphate to generate the classic second messengers inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (1). IP3 subsequently promotes calcium (Ca2+) release from the endoplasmic reticulum, whereas diacylglycerol stimulates protein kinase C (PKC), which ultimately increases Ca2+ influx through voltage-dependent L-type Ca2+ channels (2). Each of these GnRH receptor–dependent Ca2+ signals participates in the activation of mitogen-activated protein kinase signaling pathways, which lead to the increased transcriptional expression of LH and FSH. Experimental evidence suggests that Jun N-terminal kinase signaling, which is essential for up-regulation of FSH expression, depends on IP3-mediated Ca2+ release from the endoplasmic reticulum. In contrast, extracellular signal-regulated kinase (ERK) signaling, which is essential for up-regulation of LH expression, requires Ca2+ influx through L-type Ca2+ channels located in the plasma membrane (2, 3). The divergence of Ca2+-dependent regulation of two distinct mitogen-activated protein kinase signaling pathways by two distinct Ca2+ signals in gonadotropes exquisitely demonstrates the functional importance of spatially restricted Ca2+ signaling.

The abbreviations used are: GnRH, gonadotropin-releasing hormone; [Ca2+]i, intracellular Ca2+ concentration; CCCP, carbonyl cyanide m-chlorophenylhydrazone; DCF, 2′,7′-dichlorodihydrofluorescein diacetate; ERK, extracellular signal-regulated kinase; FSH, follicle-stimulating hormone; H2O2, hydrogen peroxide; IP3, inositol 1,4,5-trisphosphate; IQR, interquartile range; LH, luteinizing hormone; nP2, Ca2+ sparklet site activity; PKC, protein kinase C; ROS, reactive oxygen species; TIRF, total internal reflection fluorescence; Alexa 555–WGA, wheat germ agglutinin–Alexa Fluor 555 conjugate; DMEM, Dulbecco’s modified Eagle’s medium; YFP, yellow fluorescent protein.
ROS promote colocalized L-type calcium channel sparklets

Using a combination of electrophysiology and total internal reflection fluorescence (TIRF) microscopy, we reported that GnRH application induces discrete sites of Ca\(^{2+}\) influx through L-type Ca\(^{2+}\) channels (Ca\(^{2+}\) sparklets) in gonadotrope-derived αT3-1 cells (4). Consistent with and expanding on prior work (2, 3), we found GnRH-dependent stimulation of L-type Ca\(^{2+}\) channel sparklets to be dependent on PKC activity. Importantly, our data also indicate that highly localized PKC-dependent L-type Ca\(^{2+}\) channel sparklet activity is necessary and sufficient for ERK activation (4). G\(_{q}\) protein–coupled receptor signaling in many cells stimulates NADPH oxidase enzyme complexes, leading to the generation of reactive oxygen species (ROS) (5). Intriguingly, generation of reactive oxygen species (ROS) (5). Intriguingly, increased oxidation within mitochondria near the cell periphery. In our study, we tested the hypothesis that intracellular ROS signaling contributes to GnRH-dependent stimulation of L-type Ca\(^{2+}\) channels in gonadotropes.

In contrast to their well-documented role in cell damage and dysfunction, ROS also operate as purposeful signaling molecules in the context of normal cell activity (7). Two major sources of ROS generation include the plasma membranes of enzyme complexes and the mitochondrial electron transport chain. In this study, we hypothesized that intracellular ROS signaling contributes to GnRH-dependent stimulation of L-type Ca\(^{2+}\) channels in gonadotropes.

Using a TIRF imaging-based approach, we found that GnRH induced highly localized, punctate sites of ROS generation in the subplasmalemmal space of gonadotropes. GnRH also increased oxidation within mitochondria near the cell periphery. ROS production in response to GnRH required contributions from NADPH oxidase and mitochondria. Consistent with our overall hypothesis, exogenous hydrogen peroxide (H\(_2\)O\(_2\)) increased L-type Ca\(^{2+}\) channel activity, whereas removal of endogenous H\(_2\)O\(_2\) with catalase abolished GnRH-dependent stimulation. Furthermore, L-type Ca\(^{2+}\) channel activity observed in response to GnRH occurred predominantly at subplasmalemmal sites enriched with mitochondria. In sum, our data support a model where GnRH-dependent stimulation of L-type Ca\(^{2+}\) channels incorporates a localized H\(_2\)O\(_2\) signaling mechanism generated by NADPH oxidase and subplasmalemmal mitochondria.

Results

To test our hypothesis that ROS signaling contributes to L-type Ca\(^{2+}\) influx through L-type Ca\(^{2+}\) channels in gonadotropes exposed to GnRH, we formulated four requisite experimental criteria. 1) Exogenous ROS must stimulate localized Ca\(^{2+}\) influx through L-type Ca\(^{2+}\) channels. 2) GnRH must increase ROS generation in the subplasmalemmal space. 3) Inhibition of endogenous ROS signaling must decrease GnRH-mediated L-type channel Ca\(^{2+}\) influx. 4) GnRH-dependent sites of Ca\(^{2+}\) influx through L-type channels must be associated with sites of ROS generation.

Primary mouse gonadotropes produce L-type Ca\(^{2+}\) channel sparklets in response to GnRH and hydrogen peroxide

To continue our investigation of L-type Ca\(^{2+}\) channel signaling in gonadotropes, we once again used a combination of electrophysiology and TIRF microscopy to visualize subplasmalemmal Ca\(^{2+}\) influx with high temporal and spatial resolution (4). To this point, we have limited our investigation of L-type Ca\(^{2+}\) channel signaling in gonadotropes to immortalized mouse gonadotrope-derived αT3-1 cells (4). αT3-1 cells were carefully selected as this cell line faithfully recapitulates proximal GnRH receptor signaling mechanisms observed in native gonadotropes (2–4, 8). However, to directly extend our findings in αT3-1 cells to native cells, we examined the effects of GnRH and H\(_2\)O\(_2\) on L-type Ca\(^{2+}\) channel sparklets in genetically labeled primary mouse gonadotrope cells (9).

In single primary mouse gonadotropes voltage-clamped at −70 mV and dialyzed with the fluorescent Ca\(^{2+}\) indicator fluo-5F, GnRH (10 nm) induced discrete sites of repetitive Ca\(^{2+}\) influx (i.e. Ca\(^{2+}\) sparklets; Fig. 1A). We quantified Ca\(^{2+}\) sparklet activity by calculating Ca\(^{2+}\) channel sparklet activity (nP\(_s\)) values for each Ca\(^{2+}\) influx site where n is the number of quantal levels observed and P\(_s\) is the probability that the Ca\(^{2+}\) channel sparklet is active (see “Experimental procedures” and Refs. 4 and 10–12). GnRH increased Ca\(^{2+}\) sparklet site activity (Fig. 1B; control median nP\(_s\) = 0.11, interquartile range (IQR) = 0.25; GnRH median nP\(_s\) = 0.32, IQR = 0.66; p < 0.05, n = 15). The occurrence of Ca\(^{2+}\) sparklets, expressed as Ca\(^{2+}\) sparklet site density (Ca\(^{2+}\) sparklet sites/μm\(^2\)), also increased following GnRH exposure (Fig. 1B; control = 0.0033 ± 0.0015 sites/μm\(^2\); GnRH = 0.019 ± 0.0035 sites/μm\(^2\); p < 0.05, n = 15).

Similar to αT3-1 cells (4), incubating primary gonadotropes with the L-type Ca\(^{2+}\) channel antagonist nicardipine (10 μM for 10 min) abolished GnRH-dependent Ca\(^{2+}\) influx (Fig. 1B; n = 19). These data demonstrate that GnRH promotes localized Ca\(^{2+}\) influx through L-type Ca\(^{2+}\) channels in primary gonadotropes by increasing the number of active Ca\(^{2+}\) sparklet sites and by increasing the activity of those sites.

The L-type Ca\(^{2+}\) channel sparklets observed in primary gonadotropes were qualitatively similar to those observed in αT3-1 cells (e.g. nP\(_s\) values, bimodal activity distribution, the quantal amplitude of single-channel Ca\(^{2+}\) influx events, and size of the Ca\(^{2+}\) sparklet itself) (4). Primary cells, however, appeared to have an average L-type Ca\(^{2+}\) channel sparklet site density ~1.5-fold greater than that of αT3-1 cells. To the best of our knowledge, these data represent the first evidence of localized L-type Ca\(^{2+}\) channel signaling in native gonadotropes and validate our use of αT3-1 cells to investigate localized Ca\(^{2+}\) signaling in gonadotropes.

To test the effect of exogenous ROS on Ca\(^{2+}\) influx, we superfused primary gonadotropes with H\(_2\)O\(_2\) (100 μM) and monitored for changes in L-type Ca\(^{2+}\) channel function. H\(_2\)O\(_2\) increased nicardipine-sensitive Ca\(^{2+}\) sparklet site activity (Fig. 1, C and D; control median nP\(_s\) = 0.060, IQR = 0.06; H\(_2\)O\(_2\) median nP\(_s\) = 0.36, IQR = 0.91; p < 0.05, n = 10). L-type Ca\(^{2+}\) channel sparklet density also increased following H\(_2\)O\(_2\) exposure (Fig. 1D; control density = 0.0047 ± 0.0017 sites/μm\(^2\); H\(_2\)O\(_2\) density = 0.016 ± 0.0024 sites/μm\(^2\); p < 0.05, n = 10).
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Figure 1. GnRH and exogenous hydrogen peroxide induce localized L-type Ca\(^{2+}\) channel influx in primary mouse gonadotrope cells. A, representative TIRF images and traces showing time courses of localized Ca\(^{2+}\) influx at the circled sites before (left) and after (right) application of GnRH (10 nM); B, plot of \(n_P\), (left) and plot of mean ± S.E. Ca\(^{2+}\) sparklet densities (Ca\(^{2+}\) sparklet sites/\(\mu m^2\); right) before and after GnRH in the absence \((n = 15)\) and the continuous presence of the L-type Ca\(^{2+}\) channel antagonist nicardipine \((10 \mu M, n = 19); raw data not shown\). The horizontal dashed gray line marks the threshold for high-activity Ca\(^{2+}\) sparklet sites \((nP, \sim 0.2; see “Experimental procedures”\). C, representative TIRF images and traces showing time courses of localized Ca\(^{2+}\) influx at the circled sites before (left) and after (right) application of H\(_2\)O\(_2\) (100 \(\mu M\)). D, plot of \(n_P\), (left) and plot of mean ± S.E. Ca\(^{2+}\) sparklet densities (Ca\(^{2+}\) sparklet sites/\(\mu m^2\); right) before and after H\(_2\)O\(_2\), in the absence \((n = 10)\) and the continuous presence of nicardipine \((10 \mu M, n = 21\); raw data not shown\). Error bars represent S.E. *\(p < 0.05\); ns, not significantly different.

Similar to GnRH, incubating primary gonadotropes with the L-type Ca\(^{2+}\) channel antagonist nicardipine \((10 \mu M\) for 10 min) abolished H\(_2\)O\(_2\)-dependent Ca\(^{2+}\) influx (Fig. 1D; \(n = 21\)). Importantly, the time course of increased L-type Ca\(^{2+}\) channel sparklet activity (within 5 min) and the average \(n_P\), and density (Ca\(^{2+}\) sparklet sites/\(\mu m^2\)) in response to H\(_2\)O\(_2\) were similar to our prior observations in \(\alpha T3-1\) cells exposed to GnRH (4). These data suggest that GnRH receptor stimulation and exogenous H\(_2\)O\(_2\) application could be regulating L-type Ca\(^{2+}\) channels through a common mechanism.

Hydrogen peroxide also evokes localized L-type channel Ca\(^{2+}\) influx in immortalized gonadotrope-derived \(\alpha T3-1\) cells

Confirming our results in primary cells, exposing single gonadotrope-derived \(\alpha T3-1\) cells to exogenous H\(_2\)O\(_2\) \((100 \mu M)\) increased Ca\(^{2+}\) sparklet site activity compared with control conditions (Fig. 2, A and B; control median \(n_P\), = 0.010, IQR = 0.016; H\(_2\)O\(_2\) median \(n_P\), = 0.25, IQR = 0.41; \(p < 0.05\), \(n = 9\)). H\(_2\)O\(_2\) also increased Ca\(^{2+}\) sparklet site density (Fig. 2, A and B; control density = 0.0024 ± 0.0008 sites/\(\mu m^2\); H\(_2\)O\(_2\) density = 0.0110 ± 0.0008 sites/\(\mu m^2\); \(p < 0.05\), \(n = 9\)). We verified that the Ca\(^{2+}\) sparklets visualized in response to H\(_2\)O\(_2\) were produced by L-type Ca\(^{2+}\) channels by pretreating cells with nicardipine \((10 \mu M\) for 10 min). In the presence of L-type Ca\(^{2+}\) channel blockade, H\(_2\)O\(_2\) exposure had no demonstrable effect on Ca\(^{2+}\) sparklet activity in \(\alpha T3-1\) cells (Fig. 2D; \(p > 0.05\), \(n = 12\)). These data demonstrate that, as in primary mouse gonadotropes, H\(_2\)O\(_2\) promotes localized Ca\(^{2+}\) influx in \(\alpha T3-1\) cells by increasing the number of active L-type Ca\(^{2+}\) channel sparklet sites and by increasing the activity of those sites.

GnRH induces punctate ROS formation in the subplasmalemmal space of \(\alpha T3-1\) cells

Next, we used TIRF microscopy to visualize changes in subplasmalemmal oxidation in \(\alpha T3-1\) cells loaded with the cell-permeant fluorescent ROS indicator 2',7'-dichlorodihydrofluorescein diacetate (DCF). External application of a physiologically relevant concentration of exogenous H\(_2\)O\(_2\) \((100 \mu M)\) \((13, 14)\) increased DCF fluorescence throughout the cell footprint visible in the TIRF field (Fig. 3A). Although the
increase in fluorescence was not entirely uniform, H$_2$O$_2$ increased the average DCF fluorescence ~2-fold (Fig. 3B; p < 0.05, n = 7). Under control conditions (i.e. baseline), our TIRF images also contained sites of highly localized DCF fluorescence (ROS puncta; Fig. 3A, circled yellow) (12, 15, 16). Following H$_2$O$_2$ application, the increase in global fluorescence often obscured the identification of designatable ROS puncta (see “Experimental procedures”). Indeed, the amplitude of the average cell fluorescence following H$_2$O$_2$ was not different from that of ROS puncta observed under control conditions or in the presence of H$_2$O$_2$ (Fig. 3B; p > 0.05, n = 7). Consistent with these observations, the ROS puncta density (ROS puncta sites/μm$^2$) before and after H$_2$O$_2$ was not different (Fig. 3C; p > 0.05, n = 7).

In contrast to application of exogenous H$_2$O$_2$, exposing cells to GnRH (10 nM) in the presence of external Ca$^{2+}$ (2 mM) had no effect on the average DCF fluorescence (Fig. 3, D and E; p > 0.05, n = 17). However, consistent with a localized ROS signaling mechanism, GnRH did increase the occurrence of ROS puncta ~4-fold (Fig. 3, D and F; density before GnRH = 0.0021 ± 0.00047 ROS puncta/μm$^2$; density after GnRH = 0.0088 ± 0.0011 ROS puncta/μm$^2$; p < 0.05, n = 17). Interestingly, in the absence of external Ca$^{2+}$ (nominally Ca$^{2+}$-free), GnRH produced no observable effect on the average DCF fluorescence or the occurrence of ROS puncta (Fig. 3, D, E, and F; p > 0.05, n = 8 cells). For our GnRH experiments (before and after) in the presence (n = 17) or absence (n = 8) of external Ca$^{2+}$, average DCF and ROS puncta fluorescence intensities were not different across groups (Fig. 3E; p > 0.05). These data demonstrate that, analogous to L-type Ca$^{2+}$ channel signaling (see Ref. 4 and below), GnRH induces localized subplasmalemmal ROS signaling in gonadotrope-derived αT3-1 cells.

**NADPH oxidase contributes to localized H$_2$O$_2$ generation in the subplasmalemmal space of αT3-1 cells**

NADPH oxidase enzyme complexes initiate oxidant signaling cascades in response to activation of Go$_q$ protein–coupled receptors (5). Therefore, we examined whether stimulation of GnRH receptors, which couple to Go$_q$ proteins, promote NADPH oxidase–dependent ROS generation in αT3-1 cells. GnRH alone increased ROS puncta densities ~4-fold (see
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Figure 3. Exogenous hydrogen peroxide and GnRH increase subplasmalemmal ROS in αT3-1 cells. A, representative TIRF images showing subplasmalemmal DCF fluorescence (indicating intracellular oxidation) in a cell before (top) and after application of H$_2$O$_2$ (100 μM; bottom). Yellow circles indicate DCF fluorescence sites that satisfy the statistical criteria for ROS puncta designation (see "Experimental procedures"). B, plot of the normalized mean ± S.E. of the average cell (left) and ROS puncta (right) DCF fluorescence (arbitrary units (AU)) before and after H$_2$O$_2$ (n = 7). C, plot of the normalized mean ± S.E. of the average cell (gray, bottom) and ROS puncta (black, top) DCF fluorescence before and after GnRH in the presence (left; n = 17) and absence (right; n = 8) of external Ca$^{2+}$. D, representative TIRF images showing subplasmalemmal DCF fluorescence before (top) and after application of GnRH (10 nM; bottom) in the presence (left) and absence (right) of external Ca$^{2+}$. E, plot of the normalized mean ± S.E. of the average cell (gray, bottom) and ROS puncta (black, top) DCF fluorescence before and after GnRH in the presence (left; n = 17) and absence (right; n = 8) of external Ca$^{2+}$. F, plot of ROS puncta densities before (open circles) and after GnRH (closed circles) in the presence (left; n = 17) and absence (right; n = 8) of external Ca$^{2+}$. Error bars represent S.E. *, p < 0.05; ns, not significantly different.

However, in cells incubated with the general NADPH oxidase inhibitor apocynin (25 μM for 10 min), GnRH (10 nM) produced no change in the occurrence of ROS puncta (Fig. 4, A and C; p < 0.05, n = 14). Apocynin incubation did not affect the average DCF fluorescence or the ROS puncta fluorescence in the presence or absence of GnRH (Fig. 4B; p < 0.05, n = 14).

Superoxide (O$_2^-$) is the _de novo_ ROS product of NADPH oxidase. However, the half-life of superoxide is extremely short due to rapid conversion to H$_2$O$_2$ and oxygen by the action of superoxide dismutase or spontaneous dismutation (7). Accordingly, we assessed the contribution of H$_2$O$_2$ to GnRH-dependent ROS puncta formation. To do so, we incubated αT3-1 cells with a membrane-permeable PEG conjugate of the H$_2$O$_2$-decomposing enzyme catalase (PEG-catalase).

Following a 10-min incubation with PEG-catalase (500 units/ml), GnRH (10 nM) failed to increase the number of observable ROS puncta (Fig. 4, D and F; p > 0.05, n = 14). PEG-catalase did not change the baseline ROS puncta density (i.e. before GnRH) and did not affect the average cell DCF fluorescence or the fluorescence of detected ROS puncta (Fig. 4E; p > 0.05). These observations suggest a potential contribution of localized H$_2$O$_2$ signaling in the subplasmalemmal space following GnRH receptor activation. Because of the promiscuity of DCF as an ROS indicator, the observed PEG-catalase sensitivity does not provide sufficient evidence to support the conclusion that H$_2$O$_2$ is directly responsible for DCF ROS puncta formation in response to GnRH. These data are, however, consistent with a recent report describing the importance of NADPH oxidase activity to the function of gonadotropes (6) and suggest that H$_2$O$_2$ produced by NADPH oxidase could contribute to L-type Ca$^{2+}$ channel stimulation by GnRH.

**NADPH oxidase-derived H$_2$O$_2$ is necessary for GnRH-dependent stimulation of L-type channel Ca$^{2+}$ influx in αT3-1 cells**

To assess the role of NADPH oxidase in stimulating L-type Ca$^{2+}$ channels in gonadotropes, we measured Ca$^{2+}$ sparklet activity in response to GnRH following inhibition of NADPH oxidase with the nonselective inhibitor apocynin and the Nox1-selective inhibitor ML171 (17, 18). Note that NADPH oxidase catalytic isoforms known to be expressed explicitly in gonadotropes include Nox1, Nox2, and the dual oxidases Duox1 and Duox2 (6). Consistent with our previous results (4), GnRH (10 nM) increased αT3-1 cell L-type Ca$^{2+}$ channel activity by increasing the number of active Ca$^{2+}$ sparklet sites and by increasing the activity at those sites (Fig. 5, A, D, and E; n = 20; p < 0.05). In contrast, preincubation of cells with apocynin
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Figure 4. NADPH oxidase and endogenous hydrogen peroxide contribute to localized GnRH-dependent ROS generation in αT3-1 cells. A and D, representative TIRF images showing punctate DCF fluorescence in αT3-1 cells before (left) and after GnRH (right) in the continuous presence of the NADPH oxidase inhibitor apocynin (25 μM) (A) and the cell-permeant H₂O₂-decomposing enzyme PEG-catalase (500 units/ml) (D). B and E, plots of the normalized average puncta density (puncta/μm²) before (left) and after GnRH (right) in the continuous presence of apocynin (n = 14) (B) and PEG-catalase (n = 14) (E). C and F, plots of ROS puncta densities before (open circles) and after GnRH (closed circles) in the presence of apocynin (n = 14) (C) and PEG-catalase (n = 14) (F). Error bars represent S.E. *p < 0.05; ns, not significantly different.

(25 μM for 10 min; n = 19) or ML171 (1 μM for 10 min; n = 6; Fig. 5B) abolished GnRH-dependent stimulation of L-type Ca²⁺ channel sparklets (Fig. 5, D and E; p > 0.05).

Application of exogenous H₂O₂ peroxide promotes localized L-type channel Ca²⁺ influx (see Figs. 1 and 2) and removal of endogenous H₂O₂ with PEG-catalase decreases GnRH-dependent ROS puncta formation (see Fig. 4). To examine the necessity of endogenous H₂O₂ for GnRH-dependent stimulation of L-type Ca²⁺ channels, we tested the effect of catalase on GnRH-dependent Ca²⁺ influx. Intracellular dialysis of cell-impermeant unmodified catalase into the cytosol, achieved by inclusion of the enzyme (500 units/ml) in our internal pipette solution, abolished the stimulatory effect of GnRH on Ca²⁺ influx (Fig. 5C). Indeed, in the presence of intracellular catalase, GnRH did not change nP₂ or density (Ca²⁺ sparklet sites/μm²) compared with control (Fig. 5, D and E; p < 0.05, n = 19). These data provide further evidence that GnRH-dependent stimulation of Ca²⁺ influx in gonadotropes involves localized generation of H₂O₂ to promote localized Ca²⁺ influx through L-type Ca²⁺ channels.

GnRH increases oxidation within subplasmalemmal mitochondria in αT3-1 cells

In addition to NADPH oxidase, the mitochondrial electron transport chain is a major source of ROS generation. Mitochondria generate ROS as a consequence of cellular respiration, and ROS production by mitochondria can change in response to cellular activity and function (19–21). To determine whether mitochondria participate in oxidant-dependent stimulation of L-type Ca²⁺ channels in gonadotropes, we examined the effects of GnRH on mitochondrial oxidative status. To do so, we incubated αT3-1 cells with the mitochondrial matrix–targeted fluorescent oxidant probe MitoSOX-Red (1 μM for 30 min) and visualized the oxidative status of subplasmalemmal mitochondria with TIRF microscopy. Indicative of increased mitochondrial oxidation, GnRH (10 nm) increased MitoSOX-Red fluorescence at discrete sites throughout the visible subplasmalemmal space of αT3-1 cells (Fig. 6, A and F; p < 0.05, n = 9).

We performed two control experiments to confirm that the observed MitoSOX-Red fluorescence involved a change in mitochondrial ROS production. For a positive control, we provoked mitochondrial ROS production with the electron transport chain complex III inhibitor antimycin (500 nm) (22, 23). We found that antimycin produced an increase in MitoSOX-Red fluorescence that was qualitatively similar to GnRH and not statistically significantly different from GnRH regarding overall fluorescence intensity (Fig. 6, B and F; p > 0.05, n = 7 cells). As a negative control, we disrupted mitochondrial ROS production by collapsing the inner mitochondrial membrane potential with carbonyl cyanide m-chlorophenylhydrazone (CCCP). Following incubation with CCCP (1 μM for 10 min), we found that neither GnRH (n = 6) nor antimycin (n = 5) produced an increase in MitoSOX-Red fluorescence (Fig. 6, C and F; p > 0.05). Similarly, preincubation with the mitochon-
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Figure 5. NADPH oxidase and endogenous hydrogen peroxide contribute to localized GnRH-dependent Ca\(^{2+}\) influx in αT3-1 cells. A–C, representative TIRF images and traces showing time courses of localized Ca\(^{2+}\) influx at the circled sites before (left) and after (right) GnRH (10 nM) in a control cell (A) and in the continuous presence of the Nox1-selective NADPH oxidase inhibitor ML171 (B; 1 μM) and the H\(_2\)O\(_2\)-decomposing enzyme catalase (C; 500 units/ml). D, plot of n\(_{\text{sp}}\) before and after GnRH in control cells (n = 20) and in the continuous presence of the Nox1-selective NADPH oxidase inhibitor ML171 (n = 6), the nonselective NADPH oxidase inhibitor apocynin (representative data not shown; n = 19), and the H\(_2\)O\(_2\)-decomposing enzyme catalase (n = 19). E, plot of mean ± S.E. Ca\(^{2+}\) sparklet densities (Ca\(^{2+}\) sparklet sites/μm\(^2\)) before and after GnRH in control cells (n = 15) and in the continuous presence of ML171 (n = 6), apocynin (representative data not shown; n = 19), and the H\(_2\)O\(_2\)-decomposing enzyme catalase (n = 19). Error bars represent S.E. *, p < 0.05; ns, not significantly different.

dria-targeted antioxidant mitoTEMPO (25 ns for 10 min) (24) prevented GnRH-dependent changes in MitoSOX-Red fluorescence (n = 12; Fig. 6, D and F). Together, these results support the conclusion that increased MitoSOX-Red fluorescence observed following GnRH exposure resulted from an increase in mitochondrial ROS production.

We found that inhibition of NADPH oxidase with apocynin abolished GnRH-dependent ROS puncta formation (see Fig. 4). Accordingly, to investigate the importance of NADPH oxidase on GnRH-dependent mitochondrial ROS production, we examined the effect of GnRH on MitoSOX-Red fluorescence in the presence of apocynin. Similar to our results with the mitochondrial uncoupler CCCP, applying GnRH (10 nM) to αT3-1 cells incubated with apocynin (25 μM for 10 min) produced no change in MitoSOX-Red fluorescence (Fig. 6, E and F; p > 0.05, n = 5). We suggest that, in αT3-1 cells, NADPH oxidase activity is necessary for GnRH-dependent induction of mitochondrial ROS generation. This hypothesis is consistent with analogous ROS-induced ROS release mechanisms described in other cell types (16, 22, 25). Importantly, we did not observe significant changes in the distribution or shape of mitochondria in the subplasmalemmal space using MitoSOX-Red across all experimental groups (p > 0.05). Thus, our data suggest that GnRH-dependent stimulation of subplasmalemmal mitochondrial ROS generation does not involve mechanisms associated with alterations in mitochondrial morphology or mitochondrial recruitment to specific subplasmalemmal sites.

**Mitochondria-derived ROS are necessary for GnRH-dependent stimulation of L-type channel Ca\(^{2+}\) influx in αT3-1 cells**

Having shown that GnRH induces oxidation within mitochondria located in the subplasmalemmal space of αT3-1 cells,
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Figure 6. GnRH promotes oxidation within subplasmalemmal mitochondria in αT3-1 cells. A and B, representative TIRF images showing subplasmalemmal MitoSOX-Red fluorescence (indicating oxidation within mitochondria) in cells before (left) and after exposure (right) to GnRH (10 nm) (A) and the mitochondrial electron transport chain complex III inhibitor antimycin (500 nm) (B). C, representative TIRF images showing subplasmalemmal MitoSOX-Red fluorescence in a cell incubated with the mitochondrial uncoupler CCCP (1 μM; 10 min) before (left) and after exposure (right) to GnRH (10 nm). D, representative TIRF images showing subplasmalemmal MitoSOX-Red fluorescence in a cell incubated with the mitochondria-targeted antioxidant mitoTEMPO (25 nM; 10 min) before (left) and after (right) exposure to GnRH (10 nm). E, representative TIRF images showing subplasmalemmal MitoSOX-Red fluorescence in a cell incubated with the nonselective NADPH oxidase inhibitor apocynin (25 μM; 10 min) before (left) and after exposure (right) to GnRH (10 nm). F, plot of normalized mean ± S.E. MitoSOX-Red fluorescence intensity (arbitrary units (AU)) in αT3-1 cells before and after GnRH (n = 9), before and after antimycin (n = 7), before and after GnRH (n = 6) and antimycin (representative data not shown, n = 5) in the continuous presence of CCCP, and before and after GnRH in the continuous presence of mitoTEMPO (n = 12) and apocynin (n = 5). Error bars represent S.E., *p < 0.05; ns, not significantly different.

To promote mitochondrial ROS generation, we exposed αT3-1 cells to the mitochondrial electron transport complex III inhibitor antimycin (500 nm). Strikingly similar to our findings with GnRH and H$_2$O$_2$ (see Figs. 2 and 5), antimycin increased localized L-type Ca$^{2+}$ channel sparklet activity and density (Fig. 7, C and E; control median $nP_s = 0.02$, IQR = 0.01; antimycin median $nP_s = 0.20$, IQR = 0.54; control density = 0.0008 ± 0.0004 sites/μm$^2$; antimycin density = 0.0048 ± 0.0008 sites/μm$^2$; $p < 0.05$, n = 14). These results indicate that mitochondrial ROS generation is sufficient for oxidative stimulation of L-type Ca$^{2+}$ channels in αT3-1 cells.

Consistent with our ROS puncta observations (Fig. 7A), we found that incubating αT3-1 cells with mitoTEMPO (25 nm for 10 min) abolished the stimulatory effect of GnRH on L-type Ca$^{2+}$ channel sparklet activity (Fig. 7, D and E; $p < 0.05$, n = 14). MitoTEMPO had no effect on baseline Ca$^{2+}$ sparklet activity in the absence of GnRH ($p > 0.05$ relative to control, n = 14). From these observations, we conclude that, in αT3-1 cells, mitochondrial ROS production is necessary for L-type Ca$^{2+}$ channel stimulation by GnRH (mitoTEMPO findings) and sufficient for oxidant-dependent stimulation (antimycin findings).

**GnRH-dependent L-type channel Ca$^{2+}$ influx occurs near subplasmalemmal mitochondria**

Our data indicate that GnRH promotes localized H$_2$O$_2$ generation by an NADPH oxidase/mitochondria-dependent mechanism. If H$_2$O$_2$ produced by this mechanism regulates
ROS promote colocalized L-type calcium channel sparklets

Figure 7. Mitochondria contribute to GnRH-dependent ROS and Ca^{2+} microdomain signaling in αT3-1 cells. A, representative TIRF images showing punctate DCF fluorescence before (left) and after GnRH (10 nM; right) in cells incubated with mitoTEMPO (25 nM; 10 min). B, plot of ROS puncta densities (ROS puncta sites/μm^2) before (open circles) and after GnRH (closed circles) in the continuous presence of mitoTEMPO (n = 12). C, representative TIRF images and traces showing time courses of localized Ca^{2+} influx at the circled sites before (left) and after (right) application of the mitochondrial electron transport chain complex III inhibitor antimycin (500 nM). D, representative TIRF images and traces showing time courses of localized Ca^{2+} influx at the circled sites before (left) and after (right) application of GnRH (10 nM) in cells incubated with the mitochondria-targeted antioxidant mitoTEMPO (25 nM; 10 min). E, plot of nP, (top) and plot of mean ± S.E. Ca^{2+} sparklet densities (Ca^{2+} sparklet sites/μm^2; bottom) before and after GnRH (left; n = 14; raw data not shown), before and after antimycin (middle; n = 14), and before and after GnRH in the continuous presence of mitoTEMPO (right; n = 7). Error bars represent S.E. *, p < 0.05; ns, not significantly different.

L-type Ca^{2+} channel activity, then Ca^{2+} influx through these channels must occur near sites of H_{2}O_{2} generation such as subplasmalemmal mitochondria. To visualize the subcellular distribution of mitochondria in αT3-1 cells, we labeled mitochondria with fluorescent mitochondrial marker MitoTracker Green (200 nM for 15 min), marked the plasma membrane with a fluorescent wheat germ agglutinin–Alexa Fluor 555 conjugate (Alexa 555–WGA; 5 μg/ml for 5 min), and imaged the resulting fluorescence with confocal microscopy. Our image stacks show that mitochondria occupied 9.86 ± 0.88% of the total cell volume in αT3-1 cells (Fig. 8, A and B; n = 6 cells) with most of the MitoTracker signal occurring distal to the plasma membrane (> 0.5 μm; Fig. 8A, panel 3, green pixels). However, we did find that 28.38 ± 6.36% of the mitochondrial volume resided in the subplasmalemmal space of these cells (Fig. 8, A, panel 3, yellow pixels, and C; n = 6 cells). These data indicate that, in αT3-1 cells, the subplasmalemmal space contains a sufficient quantity of mitochondria to support ROS-dependent stimulation of L-type Ca^{2+} channels.

We then used TIRF microscopy to visualize the spatial relationship between subplasmalemmal mitochondria and L-type Ca^{2+} channel activity. To do so, we imaged L-type Ca^{2+} channel sparklets (with fluo-5F as before) in voltage-clamped αT3-1 cells loaded with MitoTracker Green (200 nM for 15 min). Our TIRF images revealed a scattered population of subplasmalemmal mitochondria (Fig. 9A, panel 1). Using thresholded MitoTracker Green images to establish clear mitochondrial boundaries, we calculated that these mitochondria occupied 13.34 ± 2.20% of the visible subplasmalemmal space (Fig. 9A, panel 2; n = 13). Exposing these cells to GnRH (10 nM) evoked L-type channel activity, then Ca^{2+} influx through these channels must occur near sites of H_{2}O_{2} generation such as subplasmalemmal mitochondria.
ROS promote colocalized L-type calcium channel sparklets

Ca\(^{2+}\) channel sparklets (Fig. 9A, panel 3) that were similar (regarding activity and density) to those observed in our previous experiments using GnRH (see Fig. 5 and Ref. 4). To quantify the spatial relationship between subplasmalemmal mitochondria and L-type Ca\(^{2+}\) channel sparklets, we overlaid our thresholded MitoTracker Green and fluo-5F images (Fig. 9A, panel 4) and measured the distance between the Ca\(^{2+}\) sparklet site peaks (pixels of highest intensity) and the edge of the nearest thresholded MitoTracker signal. Mitochondria-associated L-type Ca\(^{2+}\) channel sparklet sites were defined a priori as those sites with peaks ≤0.5 μm from the edge of the nearest thresholded MitoTracker signal. This boundary is represented by the vertical dashed gray line in Fig. 9B. By plotting the cumulative values of these distances, we found that GnRH-dependent L-type Ca\(^{2+}\) channel sparklets associate with subplasmalemmal mitochondria (Fig. 9B; n = 13 cells). Indeed, the half-distance of Ca\(^{2+}\) sparklet sites observed (n = 26 sites) to the nearest mitochondria was less than that of 130 randomly selected points within the visible plasma membrane (0.64 μm, 95% confidence interval (0.54, 0.74) for observed Ca\(^{2+}\) sparklet sites; 2.78 μm, 95% confidence interval (2.69, 2.87) for 130 random points).

To further quantify the relationship between subplasmalemmal mitochondria and L-type Ca\(^{2+}\) channel function, we compared the np\(_{\text{avg}}\) of GnRH-dependent Ca\(^{2+}\) sparklet sites associated with mitochondria (peak distance ≤ 0.5 μm) with those not associated with mitochondria (peak distance > 0.5 μm). We found that GnRH-dependent L-type Ca\(^{2+}\) channel sparklet activity at mitochondria-associated sites (median np\(_{\text{avg}}\) = 0.72, IQR = 0.88) was ~3-fold greater than that of Ca\(^{2+}\) sparklet sites not associated with mitochondria (median np\(_{\text{avg}}\) = 0.24, IQR = 0.20; Fig. 9C; n = 13 cells, p < 0.05). From these data, we conclude that, in αT3-1 cells, the spatial distribution of GnRH-dependent L-type Ca\(^{2+}\) channel activity correlates strongly with the presence of subplasmalemmal mitochondria. This conclusion supports our overall hypothesis that localized ROS generation in response to GnRH receptor stimulation promotes the opening of nearby L-type Ca\(^{2+}\) channels in gonadotropes.

Discussion

Herein, we investigated the hypothesis that localized L-type channel Ca\(^{2+}\) influx in GnRH-stimulated gonadotropes involves an intracellular oxidant signaling mechanism. Our key observations in support of this supposition are: 1) exposing primary and immortalized mouse gonadotropes to exogenous ROS (H\(_2\)O\(_2\)) provoked localized sites of L-type channel Ca\(^{2+}\) influx, 2) applying GnRH induced discrete sites of ROS generation in the subplasmalemmal space, 3) GnRH induced oxidation within subplasmalemmal mitochondria, 4) inhibiting NADPH oxidase and mitochondria-dependent H\(_2\)O\(_2\) generation abolished GnRH-dependent stimulation of L-type Ca\(^{2+}\) channels, and 5) the spatial distribution of GnRH-dependent L-type Ca\(^{2+}\) channel activity correlated with the observed incidence of subplasmalemmal mitochondria. Considering these and other findings, we conclude that in mouse gonadotropes GnRH-dependent Ca\(^{2+}\) influx through L-type Ca\(^{2+}\) channels entails a localized H\(_2\)O\(_2\)-generating mechanism with synergistic contributions from NADPH oxidase and subplasmalemmal mitochondria (Fig. 10).

Immortalized αT3-1 cells recapitulate many properties attributed to primary gonadotropes (8, 28, 29). However, it is possible that the localized L-type Ca\(^{2+}\) channel function observed in these cells (see Ref. (4) is nonrepresentative of L-type Ca\(^{2+}\) channel function in native cells. We addressed this important issue directly by examining the effect of GnRH on Ca\(^{2+}\) influx in genetically labeled primary mouse gonadotropes (9). Similar to αT3-1 cells, GnRH induced discrete sites of Ca\(^{2+}\) influx through L-type Ca\(^{2+}\) channels (Ca\(^{2+}\) sparklets). Importantly, other than a higher overall occurrence, the L-type Ca\(^{2+}\) channel sparklets we observed in primary gonadotropes were indistinguishable from those observed in αT3-1 cells.

With a sampling frequency of 50 Hz, short-lived Ca\(^{2+}\) sparklet events are likely under-represented in our recordings. However, the degree of Ca\(^{2+}\) influx associated with these brief events is relatively minor compared with that associated with the longer-lasting Ca\(^{2+}\) sparklets observed following cell stimulation (see Figs. 1 and 2 and Refs. 4 and 11). Indeed, we previously established that evoked L-type Ca\(^{2+}\) channel sparklets are biologically relevant Ca\(^{2+}\) signals in αT3-1 gonadotropes by showing a strong correlation between the Ca\(^{2+}\) sparklet activity observed (at 50 Hz) and ERK activation (4, 30). Thus, we suggest...
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Figure 9. GnRH-dependent L-type Ca\(^{2+}\) channel activity correlates with the presence of subplasmalemmal mitochondria in αT3-1 cells. A, representative TIRF images showing subplasmalemmal mitochondria (MitoTracker fluorescence, panel 1; thresholded MitoTracker fluorescence, panel 2), GnRH-dependent (10 nM) L-type channel Ca\(^{2+}\) influx (flu-5F fluorescence; panel 3), and an overlay of panels 2 and 3 (panel 4). Yellow circles in panels 3 and 4 indicate sites of L-type Ca\(^{2+}\) channel sparklet activity. B, distance map showing cumulative distribution functions representing the distance of observed Ca\(^{2+}\) sparklet site peaks from mitochondria (solid black line; n = 13) and 130 randomly distributed points (10 points per cell) within the visible TIRF footprint (dashed black line). Solid red lines are best fits of the cumulative distributions with a single exponential function (see “Experimental procedures”). The vertical dashed gray line marks the distance separating mitochondrial-associated (≤0.5 μm) and nonassociated (>0.5 μm) Ca\(^{2+}\) sparklet sites. C, plot of nP\(_{\text{a}}\) at sites >0.5 and ≤0.5 μm from the nearest thresholded MitoTracker signal (n = 13). *, p < 0.05.

Figure 10. Working model of GnRH-dependent hydrogen peroxide and Ca\(^{2+}\) microdomain signaling in anterior pituitary gonadotropes. Our data, in conjunction with previous reports (2–4), support a localized mechanism of oxidant-dependent L-type Ca\(^{2+}\) channel signaling in gonadotropes. GnRH receptor stimulation promotes the generation of NADPH oxidase-dependent H\(_2\)O\(_2\), microdomains functionally coupled (via PKC) to L-type Ca\(^{2+}\) channels. Colocalized L-type channel Ca\(^{2+}\) influx, in turn, promotes ERK activation and ultimately an increase in gonadotropin biosynthesis. Furthermore, our data suggest that mitochondria-dependent ROS-induced ROS release (RIRR) serves as an associated amplification mechanism that is necessary for the generation of functionally relevant colocalized Ca\(^{2+}\) and H\(_2\)O\(_2\) signaling microdomains. GnRHR, gonadotropin releasing hormone receptor; LTCC, L-type Ca\(^{2+}\) channel; ETC, mitochondrial electron transport chain. See text for further details.

that although a portion of the shorter-lived Ca\(^{2+}\) influx events may not be apparent in our recordings, this will minimally impact interpretation of our data due to the small contribution of missed events to total Ca\(^{2+}\) influx.

Our data show that inhibition of ROS production by either NADPH oxidase (with apocynin or ML171) or by mitochondria (with mitoTEMPO) is in each case sufficient for preventing ROS puncta formation or L-type Ca\(^{2+}\) channel signaling in response to GnRH. From an unbiased teleological perspective, either source alone or both in tandem could initiate ROS generation following GnRH receptor stimulation. However, studies on the angiotensin II type 1 receptor, which also couples to Go\(_{\alpha}\) proteins, conclude that ROS generation by NADPH oxidase precedes and induces subsequent mitochondrial ROS generation through a mechanism descriptively termed ROS-induced ROS release (4, 9, 20, 21). Accordingly, our group found that, in arterial smooth muscle cells exposed to angiotensin II, subplasmaemmal mitochondria function as amplifiers of localized oxidant signaling microdomains initiated by NADPH oxidase (16). Although data presented here are consistent with an analogous ROS-induced ROS release mechanism in gonadotropes, additional experimentation is necessary to confirm the importance of ROS-induced ROS release in gonadotropes.

Similar to our findings with GnRH, we showed that localized angiotensin II–dependent ROS generation promoted colocalized Ca\(^{2+}\) influx through L-type Ca\(^{2+}\) channels (12, 15, 16). The
stimulatory effect of angiotensin II required oxidant-depend-
ent activation of PKCα (12, 31–33). Our prior work on
gonadotropes revealed that GnRH-dependent stimulation of
L-type Ca2+ channels also required PKC with the novel δ and ε
isoforms identified as likely candidates (4). Importantly, similar
to the conventional isoforms (e.g. PKCa), the two pairs of zinc
fingers located in the regulatory domains of novel PKC iso-
forms are subject to oxidation, thus rendering these kinases sus-
tceptible to activation by ROS (32, 34). Thus, given our find-
ings that PKC activation and ROS generation are each required
for GnRH-dependent stimulation of L-type Ca2+ channels in
gonadotropes, it is conceivable, if not likely, that ROS-depen-
dent regulation of PKC activity occurs in these cells. Future
experimentation is necessary to confirm the occurrence and
significance of ROS-dependent regulation of PKC activity in
gonadotropes.

Analogous to our angiotensin II–related findings in arterial
smooth muscle (12, 15, 16), the observed incidence of punctate
DCF fluorescence in gonadotropes occurs only after cell stim-
ulation (e.g. GnRH). Similarly, ROS puncta generation in
response to GnRH was ablated by inhibiting endogenous ROS
generation by NADPH oxidase (with apocynin and ML171) and
mitochondria (with mitoTEMPO) and by disrupting H2O2 sig-
naling with exogenous catalase. Together, these findings indi-
cate that our results obtained with DCF require biologically
relevant ROS generation and that some of the properties of
localized ROS signaling in these two disparate cell types are
shared.

However, in contrast to our findings in smooth muscle where
the absence of external Ca2+ did not influence ROS puncta
formation, our results from gonadotropes indicate that GnRH-
dependent ROS generation requires external Ca2+. The under-
lying basis for the difference in Ca2+ dependence is unclear.
The mechanisms by which Ca2+ contributes to GnRH-depen-
dent ROS generation are, in general, not understood. Evidence
suggests that Ca2+ increases ROS signaling in gonadotropes by
activating NADPH oxidase complexes containing Ca2+-respon-
sive Duox catalytic subunits (6). However, indicating a role
for Nox1-containing complexes, we found that inhibition of
NADPH oxidase with the Nox1-selective inhibitor ML171 pre-
vented GnRH-dependent stimulation of L-type Ca2+ channels.
Thus, further examination of NADPH oxidase signaling com-
plexes in gonadotropes is warranted. Ca2+ influx could also
increase ROS generation by NADPH oxidase complexes by
activating PKC (5). Alternatively, elevated Ca2+ within the
mitochondrial matrix promotes ROS generation by increasing
the activity of citric acid cycle dehydrogenases and possibly the
respiratory chain directly (35, 36). Irrespective of mechanism,
our data showing ROS-dependent Ca2+ influx and Ca2+-depen-
dent ROS generation in gonadotropes indicate that GnRH
induces a self-amplifying signaling unit via a reciprocal cou-
pling mechanism with two outputs (i.e. Ca2+ and ROS).

ROS generation by NADPH oxidase and mitochondria
essentially begins with the formation of superoxide (O2-) (5, 7,
25). However, spontaneous or catalytic dismutation of super-
oxide to H2O2 and O2, along with other inherent biochemical
constraints, limits the biological utility of superoxide as a dif-
fusible second messenger (5, 7, 25). Accordingly, in the context
of oxidant-dependent signal transduction, evidence suggests
that superoxide-derived H2O2 serves as the major functional
ROS signaling intermediate. Our catalase data are consistent
with H2O2 playing an essential role in oxidant-dependent stim-
ulation of L-type Ca2+ channels in gonadotropes. Incubating
gonadotropes with cell-permeable PEGylated catalase pre-
vented GnRH from increasing the occurrence of ROS puncta,
and dialyzing cells with catalase abolished the stimulatory effect
of GnRH on L-type Ca2+ channel function. Thus, our results
indicate that GnRH promotes localized subplasmalemmal
H2O2 generation in gonadotropes.

To conclude, our data support a conceptual model in
gonadotropes where GnRH receptor activation promotes a
localized subplasmalemmal ROS signaling mechanism medi-
ated by H2O2 initiated by NADPH oxidase, and amplified by
mitochondria that in turn stimulates localized Ca2+ influx
through L-type Ca2+ channels (Fig. 10). Although several
mechanistic uncertainties require further investigation, our
functionally coupled Ca2+ and ROS microdomain model im-
plies that reciprocal modulation of L-type Ca2+ channels and
NADPH oxidase and mitochondrial ROS generation extend
beyond simple colocalization of two signaling mechanisms. For
example, our model suggests potential perturbation of numer-
ous cellular processes by conditions such as metabolic dysfunc-
tion (e.g. obesity and diabetes) by influencing the availability
of the reducing equivalent NADPH or by altering the bioenergetic
activity of subplasmalemmal mitochondria. Thus, continued
characterization of Ca2+ and ROS signaling mechanisms will
impact the discovery of novel therapeutic strategies to manip-
ulate GnRH receptor function in the management of fertility,
obesity-related endocrine dysfunction (37), polycystic ovarian
syndrome (38, 39), and GnRH-sensitive carcinomas (40–42).

Experimental procedures

Materials and methods

We purchased Dulbecco’s modified Eagle’s medium
(DMEM) from HyClone (Logan, UT), fetal bovine serum from
Atlas Biologicals (Fort Collins, CO), Matrigel from BD Biosci-
cences, and mitoTEMPO from Enzo (Farmingdale, NY). Cata-
lase and papain were from Worthington Biochemical, and we
purchased ML171 from Tocris (Minneapolis, MN). Fluoro-5F
(pentapotassium salt), DCF, Alexa 555–WGA, MitoSOX-Red,
MitoTracker Green, and sodium pyruvate were from Invitro-
gen. All other chemicals were purchased from Sigma.

Cell culture

Clonal αT3-1 mouse gonadotrope cells (8), a generous gift
from Dr. Pamela Mellon (University of California, San Diego),
were incubated in high-glucose DMEM supplemented with
fetal bovine serum (10%) and sodium pyruvate (1 mM). Cells
were maintained at 37 °C in 5% CO2 humidified air.

Mouse pituitary cell dissociation and gonadotrope isolation

Primary cells were from transgenic mice with genetically
labeled fluorescent gonadotropes produced by a cross of GnRH
receptor promoter–driven Cre recombinase (GRIC) mice with
ROSA26-YFP or ROSA-tdTomato mice (9). Sexually mature
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(6–12 weeks of age) male and female mice were euthanized with sodium pentobarbital (200 mg/kg intraperitoneally) in strict accordance with institutional guidelines and with approval by the Institutional Animal Care and Use Committee of Colorado State University. Removed pituitaries were enzymatically digested in Ca2+-free buffer containing papain (10 units/ml) and DTT (1 mg/ml) for 15 min at 37 °C followed by a second incubation (15 min at 37 °C) in Ca2+-free buffer supplemented with collagenase (300 units/ml, type II). Digested pituitary tissue was then washed with and placed in high-glucose DMEM for 30 min after which trituration with a fire-polished Pasteur pipette was used to create a cell suspension for plating. Cells plated on poly-l-lysine–coated MatTek dishes were incubated overnight at 37 °C in 5% CO2 humidified air and used for experimentation within 24–36 h. YFP- and tdTomato-labeled gonadotrope knockin mice were used to ensure consistency among mouse lines. Significant differences between groups were not observed; thus, data from the two groups were combined for analysis.

Detection of ROS generation

TIRF microscopy was used to visualize subplasmalemmal ROS generation as described previously (12, 15). Briefly, we incubated αT3-1 cells in Ca2+-free buffer supplemented with the cell-permeant ROS indicator DCF (1 μM) for 30 min at room temperature (22–25 °C). Cells were then placed in either Ca2+-free or Ca2+-containing (2 mM) buffer for experimentation following removal of excess DCF. A 491 nm laser provided excitation of oxidized DCF; appropriate filters separated excitation and emission light. Due to the lack of specificity of DCF as an ROS indicator, increases in DCF fluorescence are interpreted only as an indication of intracellular oxidation without reference to specific oxidants (e.g. H2O2).

For an area of elevated DCF fluorescence to be considered a site of increased ROS generation (a ROS “punctum”), a grid of 3 × 3 contiguous pixels had to have a fluorescence amplitude equal to or larger than the mean basal DCF fluorescence plus 3 times the standard deviation (10, 12). We calculated the density of ROS puncta (ROS puncta/μm2) by dividing the number of sites detected by the area of cell membrane visible in the TIRF images. For consistency and to ensure proper cell loading with DCF, cells chosen for analysis had one to two visible ROS puncta at baseline and showed changes in DCF fluorescence only in response to cell stimulation. We calculated changes in DCF fluorescence (∆DCF) from the mean pixel intensities of the total intracellular submembranous slice visible in the TIRF images (average ∆DCF) and the areas confined to identified ROS puncta (puncta ∆DCF).

Electrophysiology and Ca2+ imaging

Simultaneous electrophysiology and TIRF Ca2+ imaging experiments were carried out using the conventional dialyzed whole-cell patch clamp technique as described previously (4, 10, 12, 15, 43). Briefly, we imaged Ca2+ influx with a TILL Photonics (FEI, Munich, Germany) through-the-lens TIRF built around an inverted Olympus IX-71 microscope (Center Valley, PA) equipped with a 100× TIRF oil-immersion objective (numerical aperture, 1.45) and an iXON electron-multiplying charge-coupled device camera (Andor Technology, South Windsor, CT). Ca2+ influx was visualized in gonadotropes using the fluorescent Ca2+ indicator fluo-5F (200 μM) and an excess of EGTA (10 mM) introduced via the patch pipette. An Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA) was used to control membrane potential, a 491 nm laser provided fluo-5F excitation, and appropriate filters separated excitation and emission light. Ca2+ influx was recorded in the presence of 2 mM external Ca2+ at a frame rate of 50 Hz and holding membrane potential of −70 mV to increase the driving force for Ca2+ entry. To preclude potential contaminating Ca2+ release events from the endoplasmic reticulum, the Ca2+-ATPase inhibitor thapsigargin (1 μM) was present in all experiments. We imaged cells for 2 min before applying GnRH (10 nM), H2O2 (100 μM), nicardipine (10 μM), apocynin (25 μM), catalase (500 units/ml), antimycin A (500 nM), or mitoTEMPO (25 nM). Imaging of all treatment conditions continued for a minimum of 10 min. We performed vehicle control experiments as appropriate; all experiments were carried out at room temperature (22–25 °C).

L-type Ca2+ channel sparklet analysis

Following background subtraction, we converted fluo-5F fluorescence signal image stacks to intracellular Ca2+ concentrations ([Ca2+]i) and analyzed these data with custom software as described previously (4, 12, 15, 43). Briefly, we quantified L-type Ca2+ channel sparklet activity by calculating the nP, of each site where n is the number of quantal levels detected and P is the probability that the site is active. nP values were obtained using pCLAMP 10.0 (Molecular Devices) on imported [Ca2+]i time course records using an initial unitary [Ca2+]i elevation of ≈20 nM as determined empirically. For an elevation in [Ca2+]i, to be considered an L-type Ca2+ channel sparklet event, a grid of 3 × 3 contiguous pixels had to have a [Ca2+]i amplitude equal to or larger than the mean basal [Ca2+]i, plus 3 times the standard deviation. Consistent with previous reports (10–12, 43), we observed a bimodal distribution of L-type Ca2+ channel sparklet activity in gonadotropes with sites of low activity (nP, between 0 and 0.2) and high activity (nP, greater than 0.2). We calculated active L-type Ca2+ channel sparklet site densities (Ca2+ sparklet sites/μm2) by dividing the number of active sites by the area of cell membrane visible in the TIRF images. Image stacks of Ca2+ sparklet activity were analyzed after a minimum of 5 min and before a maximum of 10 min following each manipulation in all experiments to maintain consistency and enhance reproducibility.

Imaging of subplasmalemmal mitochondria

We used TIRF microscopy to image subplasmalemmal mitochondria in αT3-1 cells. Briefly, cells were incubated with MitoTracker Green (200 nM) for 15 min in Ca2+-free buffer at 37 °C. Subplasmalemmal MitoTracker Green was excited with a 491 nm laser; appropriate filters separated excitation and emission light. For an area of elevated MitoTracker fluorescence to be considered indicative of subplasmalemmal mitochondria, the fluorescence amplitude had to be equal to or larger than the mean basal fluorescence plus 3 times the standard deviation. Using this criterion, we generated thresholded MitoTracker
TIRF images to establish clear mitochondrial boundaries. We calculated the percentage of plasma membrane associated with subplasmalemmal mitochondria by dividing the area of mitochondria-associated membrane by the area of plasma membrane visible in the TIRF field.

For the experiments where we imaged subplasmalemmal mitochondria (first) and Ca\(^{2+}\) influx (second), we defined mitochondria-associated L-type Ca\(^{2+}\) channel sparklet sites as those sites with peaks (pixels of highest intensity) less than or equal to 0.5 \(\mu\text{m}\) from the edge of the nearest thresholded MitoTracker signal. Euclidean distance mapping analysis was used to quantify the distance of observed Ca\(^{2+}\) sparklet site peaks from the nearest thresholded MitoTracker signal and 130 randomly distributed points located within the visible TIRF footprint of the cells analyzed. Each cumulative distribution was fit with a single exponential function, \(Y = Y_o + (\text{plateau} - Y_o) \times [1 - \exp(-\ln2/X_{0.5}) \times X]]\) where \(Y\) is the cumulative frequency, \(Y_o\) is the \(Y\) value when \(X\) (distance) is zero, plateau is the \(Y\) value at infinite times, and \(X_{0.5}\) (half-distance) is \(X\) where 50% of the \(Y\) values are distal to \(X = 0\).

**Mitochondrial oxidation measurements**

TIRF microscopy was used to assess the relative oxidative status within subplasmalemmal mitochondria in αT3-1 cells using the cell-permeant fluorescent mitochondrial indicator MitoSOX-Red. We incubated αT3-1 cells in Ca\(^{2+}\)-free buffer supplemented with MitoSOX-Red (1 \(\mu\text{M}\)) for 30 min at 37 °C. A 491 nm laser provided MitoSOX-Red excitation; appropriate filters separated excitation and emission light. Cells were exposed to GnRH (10 nM) or antimycin A (500 nM) with or without pretreatment (10 min) with CCCP (1 \(\mu\text{M}\)) or apocynin (25 \(\mu\text{M}\)). We analyzed MitoSOX-Red fluorescence intensity (indicative of mitochondrial oxidation) on background-subtracted images. For an area of elevated MitoSOX-Red fluorescence to be considered indicative of oxidation within subplasmalemmal mitochondria, the fluorescence amplitude had to be equal to or larger than the mean basal fluorescence plus 3 times the standard deviation. Using this criterion, we identified normalized regions of interest to compare mitochondrial oxidation before and after treatments by comparing the fluorescence intensity. The cell outline (*white dotted line*) was generated by the custom software used to identify cellular borders visible in the TIRF field for Ca\(^{2+}\) sparklet analyses (4, 12, 15, 43).

**Confocal microscopy**

We used laser-scanning confocal microscopy to image plasma membranes and mitochondria of αT3-1 cells. The extracellular face of the plasma membrane was marked with Alexa 555–WGA (5 \(\mu\text{g}/\text{ml}\) in Ca\(^{2+}\)-free buffer for 5 min at room temperature. Mitochondria were labeled with Mito-Tracker Green (200 nm for 15 min) in Ca\(^{2+}\)-free buffer at 37 °C. Alexa Fluor 555 was excited with a 543 nm laser, and Mito-Tracker Green was excited with a 488 nm laser; appropriate filters separated excitation and emission light. We analyzed data with Volocity 3D Image Analysis Software (PerkinElmer Life Sciences). Mitochondria-associated fluorescence located ≤0.5 \(\mu\text{m}\) from the center of the Alexa 555–WGA signal was designated as peripheral.

**Statistics**

Statistical analyses were performed using GraphPad Prism 6 software. We have presented normally distributed data as the mean ± S.E. Two-sample comparisons of these data were performed using either a paired or unpaired (as appropriate) two-tailed Student’s t test, and comparisons between more than two groups were performed using a one-way analysis of variance with Tukey’s multiple comparison post-test. L-type \(n_P\) data sets were bimodally distributed (10, 12, 15). Thus, we performed two-sample comparisons of \(n_P\) data using the nonparametric Wilcoxon–Mann–Whitney test (two-tailed), whereas comparisons between more than two groups were performed using the nonparametric Friedman test with Dunn’s multiple comparison post-test. Arithmetic means of \(n_P\) data sets are indicated in the figures (*solid gray horizontal lines*) for nonstatistical visual purposes, and *dashed gray lines* mark the threshold for high-activity Ca\(^{2+}\) sparklet sites (\(n_P \geq 0.2\)) (10, 12, 43). A \(p\) value of <0.05 was considered significant, and asterisks (*) used in the figures are included to indicate significance, \(n\) indicates not significantly different, and \(n\) indicates the number of independent experiments performed on single cells unless stated otherwise.

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