Mitochondria Shape Hormonally Induced Cytoplasmic Calcium Oscillations and Modulate Exocytosis*

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Pituitary gonadotropes transduce hormonal input into cytoplasmic calcium ([Ca\(^{2+}\)]\(_{\text{cyt}}\)) oscillations that drive rhythmic exocytosis of gonadotropins. Using Calcium Green-1 and rhod-2 as optical measures of cytoplasmic and mitochondrial free Ca\(^{2+}\), we show that mitochondria sequester Ca\(^{2+}\) and tune the frequency of [Ca\(^{2+}\)]\(_{\text{cyt}}\) oscillations in rat gonadotropes. Mitochondria accumulated Ca\(^{2+}\) rapidly and in phase with elevations of [Ca\(^{2+}\)]\(_{\text{cyt}}\) after GnRH stimulation or membrane depolarization. Inhibiting mitochondrial Ca\(^{2+}\) uptake by the protonophore CCCP reduced the frequency of GnRH-induced [Ca\(^{2+}\)]\(_{\text{cyt}}\) oscillations or, occasionally, stopped them. Much of the Ca\(^{2+}\) that entered mitochondria is bound by intramitochondrial Ca\(^{2+}\) concentration; GnRH, gonadotropin-releasing hormone; [Ca\(^{2+}\)]\(_{\text{cyt}}\), cytoplasmic calcium concentration; CCCP, carbonyl cyanide m-chlorophenyl hydrazone; TMRE, tetramethylrhodamine ethyl ester. This paper is available on line at http://www.jbc.org

A mitochondrial contribution to intracellular Ca\(^{2+}\) dynamics has been debated for several decades. For some time it was supposed that mitochondria do not accumulate significant Ca\(^{2+}\) unless the surrounding free cytoplasmic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_{\text{cyt}}\)) is dangerously high (1, 2). This view seemed plausible because accumulating Ca\(^{2+}\) would divert energy of the mitochondrial membrane potential from its normal function of producing ATP, and, because the endoplasmic reticulum is already specialized for sequestering and releasing Ca\(^{2+}\), regulation by yet another organelle seemed unnecessary. Nevertheless, in some cells, mitochondria do take up Ca\(^{2+}\) at apparently physiological [Ca\(^{2+}\)]\(_{\text{cyt}}\) levels (<2 μM) (Refs. 3–11; for reviews see Refs. 12–14).

Anterior pituitary gonadotropes secrete gonadotropins in response to gonadotropin-releasing hormone (GnRH). Binding of GnRH to its cell surface receptors induces [Ca\(^{2+}\)]\(_{\text{cyt}}\) oscillations, driven primarily by the release and re-uptake of Ca\(^{2+}\) from inositol 1,4,5-trisphosphate-sensitive Ca\(^{2+}\) stores (for reviews see Refs. 15–17). These [Ca\(^{2+}\)]\(_{\text{cyt}}\) oscillations trigger secretion of luteinizing hormone and follicle-stimulating hormone, which are released into the circulation as [Ca\(^{2+}\)]\(_{\text{cyt}}\) rises transiently and repetitively above 1 μM. Local [Ca\(^{2+}\)]\(_{\text{cyt}}\) may become even higher at sites of secretion (18). Because this dynamic behavior is almost exclusively under control of intracellular organelles, gonadotropes are ideal for examining the relative contribution of intracellular organelles, including mitochondria (19), to Ca\(^{2+}\) balance during a physiological response.

Cytosolic Ca\(^{2+}\) oscillations in gonadotropes have been fairly well described with a model (20) that assumes only two Ca\(^{2+}\) compartments: endoplasmic reticulum and cytosol. The IP\(_3\) receptor/Ca\(^{2+}\) channels on the endoplasmic reticulum are first activated by elevated levels of IP\(_3\), releasing Ca\(^{2+}\) into the cytosol, and then the IP\(_3\) receptor/Ca\(^{2+}\) channels are inhibited (21) by the elevated [Ca\(^{2+}\)]\(_{\text{cyt}}\). With these Ca\(^{2+}\) channels closed, Ca\(^{2+}\) is pumped back into the endoplasmic reticulum, allowing the channels to recover, and the cycle repeats. The following observations from our laboratory suggest, however, that at least one other compartment is involved: (i) the clearance of Ca\(^{2+}\) from the cytoplasm has an unusual multicomponent time course (22), (ii) [Ca\(^{2+}\)]\(_{\text{cyt}}\) clearance was faster than Ca\(^{2+}\) reappearance in the endoplasmic reticulum measured with compartmentalized dye (23), and (iii) mitochondrial inhibitors may block Ca\(^{2+}\) oscillations induced by GnRH (19). Principally by using compartmentalized fluorescent indicators to measure [Ca\(^{2+}\)]\(_{\text{cyt}}\) and [Ca\(^{2+}\)]\(_{\text{mito}}\) simultaneously, this work evaluates quantitatively whether mitochondria are significant players in the cycling of Ca\(^{2+}\) during hormone-induced Ca\(^{2+}\) oscillations.

EXPERIMENTAL PROCEDURES

Pituitary Cell Isolation—Anterior pituitary glands were removed from 4–6-week-old male rats that had been castrated at week 2 by the supplier. Rats were euthanized by placing them in an empty chamber that was then filled with CO\(_2\). Tissue was enzymatically dispersed (24), and cells were cultured for up to 4 days.

[Ca\(^{2+}\)]\(_{\text{cyt}}\) Measurements—The loading of membrane-permeant Ca\(^{2+}\) indicators, and subsequent photometry was described previously (8). In experiments where mitochondrial membrane potential was monitored, cells were incubated in TMRE (600 nm) for 5 min following loading with Ca\(^{2+}\) indicators, and TMRE (100 nm) was present in the bath throughout the experiment. Corrections were applied to fluorescence signals to
account for bleach and bleed-through of Calcium Green-1 fluorescence into the long wavelength detection channel (8). Fluorescence intensities were converted into free \([Ca^{2+}]\) as described previously (25). Data are presented as free \([Ca^{2+}]\) in nanomolar, or as \(\Delta F/F_0\), the change in fluorescence \(\Delta F\) divided by the basal fluorescence \(F_0\).

A correction was also made for the fraction of rhod-2 in the cytoplasm. The \([Ca^{2+}]_{cyt}\) was calculated from the Calcium Green-1 fluorescence signal. Then a hypothetical cytoplasmic rhod-2 fluorescence was calculated using the time course of \([Ca^{2+}]_{cyt}\) determined from Calcium Green-1. The measured rhod-2 signal was considered to be composed of a cytoplasmic part, behaving as described above, and the mitochondrial part. The assumed fraction of cytoplasmic rhod-2 was then adjusted manually until the corrected rhod-2 fluorescence trace lost the rapid oscillatory falling components that coincided with those seen in the cytoplasmic Calcium Green-1 signal. The dissociation constants for \(Ca^{2+}\) binding to Calcium Green-1 and rhod-2 used for this correction were 190 and 570 nm respectively, and the ratios of maximum to minimum fluorescence, 7.8 and 38, respectively, were determined empirically. The fraction of rhod-2 that remained in the cytoplasm, by this minimum fluorescence, 7.8 and 38, respectively, were determined empirically. The fraction of cytoplasmic rhod-2 was then adjusted empirically. The fraction of rhod-2 that remained in the cytoplasm, by this minimum fluorescence, 7.8 and 38, respectively, were determined empirically. The fraction of cytoplasmic rhod-2 was then adjusted empirically. The fraction of rhod-2 that remained in the cytoplasm, by this minimum fluorescence, 7.8 and 38, respectively, were determined empirically. The fraction of cytoplasmic rhod-2 was then adjusted empirically. The fraction of rhod-2 that remained in the cytoplasm, by this minimum fluorescence, 7.8 and 38, respectively, were determined empirically. The fraction of cytoplasmic rhod-2 was then adjusted empirically. The fraction of rhod-2 that remained in the cytoplasm, by this minimum fluorescence, 7.8 and 38, respectively, were determined empirically. The fraction of cytoplasmic rhod-2 was then adjusted empirically. The fraction of rhod-2 that remained in the cytoplasm, by this minimum fluorescence, 7.8 and 38, respectively, were determined empirically. The fraction of cytoplasmic rhod-2 was then adjusted empirically.

The hypothesis was that a significant fraction of the cellular \([Ca^{2+}]\) would be taken up into mitochondria during a period of GnRH-induced \([Ca^{2+}]\) oscillations. To block this hypothesized mitochondrial contribution to \([Ca^{2+}]_{cyt}\), we added the protonophore CCCP, which should collapse the mitochondrial membrane potential and eliminate the electrical driving force for mitochondrial \([Ca^{2+}]\) uptake. Fig. 1 shows that application of CCCP (2 \(\mu\)M) during periodic \([Ca^{2+}]\) oscillations dramatically slowed the rate of each downstroke of \([Ca^{2+}]_{cyt}\) and reduced the frequency of oscillation. In this experiment, the rate of fall was 781 \(\pm\) 43 \(\text{nm s}^{-1}\) \((n = 7)\) in the absence of CCCP and 391 \(\pm\) 47 \(\text{nm s}^{-1}\) \((n = 4)\) in its presence. In five fully analyzed experiments, the falling phase was slowed 40 \(\pm\) 6\% by adding CCCP. A similar effect was observed in 12 out of 15 cells tested in this manner. In the remaining three cells, CCCP treatment stopped \([Ca^{2+}]_{cyt}\) oscillations completely. In either case, the effect of protonophore was reversible within 30–40 s of washout. The cell in Fig. 1 was exposed to oligomycin-B (2.5 \(\mu\)M) throughout to inhibit the mitochondrial F1-F0 ATPase and thus prevent mitochondrial ATP consumption during CCCP application. Similar results were obtained with or without oligomycin-B, suggesting that the effects of protonophore are not due to a reduction in cytoplasmic ATP. Apparently energized mitochondria mediate about 40\% of the \(Ca^{2+}\) clearance that terminates each cycle of physiological \(Ca^{2+}\) oscillations.

**Mitochondria Accumulate \(Ca^{2+}\) during Cytosolic Oscillations—** Accumulation of \(Ca^{2+}\) in mitochondria can be measured with \(Ca^{2+}\)-sensitive dyes trapped in the mitochondria. Membrane-permeant rhod-2-acetoxyethyl ester has a net positive charge and distributes preferentially into energized mitochondria where it is cleaved and made membrane-impermeant by endogenous esterases (8, 29). Fig. 2 shows a merged confocal image of a gonadotrope co-loaded with the acetoxyethyl ester forms of Calcium Green-1 and rhod-2. Calcium Green-1 was distributed diffusely in the cytosol, whereas rhod-2 had a more punctate, compartmentalized distribution, as expected from mitochondria. With cells loaded in this way, we could monitor changes of \([Ca^{2+}]_{cyt}\) in the cytosol and in the mitochondria simultaneously. Fig. 3, shows a Calcium Green-1 trace (bottom panel) and two versions of the rhod-2 trace (top panel) during a GnRH application. One rhod-2 trace (A) is corrected for spectral bleed-through (50\%) of the Calcium Green-1 fluorescence into the rhod-2 detection band, and the second trace (B) is corrected both for bleed-through and for the fraction of rhod-2 dye estimated to be in the cytosol (35\% here; see “Experimental Procedures”). As expected, GnRH (0.5 \(\text{nm}\)) induced \([Ca^{2+}]_{cyt}\) oscillations. The \([Ca^{2+}]_{cyt}\) rose simultaneously with global \([Ca^{2+}]_{cyt}\).
Mitochondria Shape Cytoplasmic Calcium Oscillations

and continued to increase over the course of 5–10 oscillations. Thus mitochondria accumulate Ca\(^{2+}\) rapidly during GnRH-induced oscillations, and \([\text{Ca}^{2+}]_{\text{mit}}\) remains elevated for many minutes even after oscillations cease and the \([\text{Ca}^{2+}]_{\text{cyt}}\) has returned to near basal levels.

\(\text{Ca}^{2+}\) Influx Depolarizes the Mitochondrial Membrane—The inner mitochondrial membrane should be transiently depolarized as \(\text{Ca}^{2+}\) flows into the mitochondria. To further test the hypothesis that \(\text{Ca}^{2+}\) enters mitochondria during GnRH-induced oscillations, we used a qualitative measure of mitochondrial membrane potential. A large matrix-negative membrane potential allows the fluorescent dye TMRE, a positively charged membrane-permeant molecule, to partition into mitochondria. At bath concentrations in the micromolar range, TMRE accumulates in mitochondria to self-quenching concentrations. As the electrical driving force for TMRE accumulation reduces during mitochondrial membrane depolarization, a fraction of the dye leaves the mitochondria, resulting in a partial relief of self-quench and an increase in fluorescence (11). Experiments using TMRE in this way showed that GnRH caused oscillations in the mitochondrial membrane potential and that the mitochondrial depolarizations coincided with each elevation in \([\text{Ca}^{2+}]_{\text{cyt}}\) (Fig. 4). Strong mitochondrial depolarization by application of the protonophore CCCP at the end of these experiments elicited a 7–8-fold larger increase in the TMRE signal. The second application was in the presence of 8 \(\mu\)M CGP-37157 (Fig. 5, gray region of trace). The CGP-37157 did not affect the rise of \([\text{Ca}^{2+}]_{\text{mit}}\), but it greatly slowed the decline as long as it was present. The apparent increase in the rate of fall of \([\text{Ca}^{2+}]_{\text{mit}}\) in the “after” trace compared with the “before” trace represents variation between trials because this phenomenon was not consistent among all of the experiments done in this way. In three other experiments in which a comparison was possible at the same \([\text{Ca}^{2+}]_{\text{mit}}\) (600 nM), CGP-37157 reduced the rate of \([\text{Ca}^{2+}]_{\text{mit}}\) fall from 8.2 ± 2 nM s\(^{-1}\) to 3.1 ± 1.2 nM s\(^{-1}\). Evidently, \(\text{Na}^{+}\)-\(\text{Ca}^{2+}\) exchange is the dominant pathway for \(\text{Ca}^{2+}\) extrusion from the mitochondria over this range of \([\text{Ca}^{2+}]_{\text{mit}}\).

**Estimation of Intramitochondrial \(\text{Ca}^{2+}\) Buffering—**Mitochondrial \(\text{Ca}^{2+}\) buffering should be critical in determining the amount of \(\text{Ca}^{2+}\) mitochondria can accumulate. We define the intramitochondrial \(\text{Ca}^{2+}\) binding ratio as the ratio of change-of-total to change-of-free mitochondrial \(\text{Ca}^{2+}\). To estimate this parameter, we compared the change of \([\text{Ca}^{2+}]_{\text{mit}}\) to the concurrent change of \([\text{Ca}^{2+}]_{\text{cyt}}\). This was done under conditions favoring \(\text{Ca}^{2+}\) movement from the cytosol to the mitochondria while maintaining other nonmitochondrial \(\text{Ca}^{2+}\) transport processes inhibited. To achieve such a state (Fig. 6A), we applied thapsigargin (1 \(\mu\)M) to inhibit \(\text{Ca}^{2+}\) uptake into the endoplasmic reticulum and CCCP (2 \(\mu\)M) to limit \(\text{Ca}^{2+}\) uptake into mitochondria. While in CCCP, the cell was briefly depolarized with 70 mM KCl to load the cytosol with \(\text{Ca}^{2+}\) and then the perfusion medium was immediately switched to one containing La\(^{3+}\), but no \(\text{Na}^{+}\) or \(\text{Ca}^{2+}\), to prevent further \(\text{Ca}^{2+}\) entry and extrusion at the plasma membrane. Mitochondria were then allowed to repolarize and accumulate \(\text{Ca}^{2+}\) by removing the CCCP, and the resulting rise in \([\text{Ca}^{2+}]_{\text{mit}}\) and fall of \([\text{Ca}^{2+}]_{\text{cyt}}\) were measured. The mitochondrial \(\text{Ca}^{2+}\) binding ratio, \(\text{CBR}_{\text{mit}}\), was calculated by the equation,

\[
\text{CBR}_{\text{mit}} = \frac{\text{CBR}_{\text{cyt}} - \frac{\Delta[\text{Ca}^{2+}]_{\text{mit}}}{\Delta[\text{Ca}^{2+}]_{\text{cyt}}} \cdot \frac{1}{F_{\text{mit}}}}
\]

\(\text{Eq. 1}\)
Mitochondria Shape Cytoplasmic Calcium Oscillations

Fig. 5. Na\(^{+}\)-Ca\(^{2+}\) exchange governs Ca\(^{2+}\) extrusion from mitochondria. The rhod-2 signal following GnRH (5 nM) stimulation was measured in the absence (before and after traces) and presence of a specific inhibitor of the mitochondrial Na\(^{+}\)-Ca\(^{2+}\) exchanger. Fluorescence records were corrected (see “Experimental Procedures”) and aligned in time to the beginning of GnRH application and normalized (ΔF/Δt) records were scaled by 0.7, 0.9, and 1.4 for before, CGP-37157, and after traces, respectively, to align the maximum fluorescence signal following GnRH removal. CGP-37157 (8 μM) was present in the perfusion solution during the time indicated by the gray portion of the trace.

where CBR\(_c\) is the cytoplasmic Ca\(^{2+}\) binding ratio (taken as 100) (31) and \(P_m\) is the mitochondrial volume as a fraction of cytoplasmic volume (assumed to be 0.02). \(\Delta[Ca^{2+}]_\text{cyt}(t_2-t_1)\) and \(\Delta[Ca^{2+}]_\text{mit}(t_2-t_1)\) represent the changes in [Ca\(^{2+}\)]\(_\text{cyt}\) and [Ca\(^{2+}\)]\(_\text{mit}\) determined during the time interval \((t_2 - t_1)\). In six experiments, the calculated binding ratio ranged widely and averaged 4027 ± 2031. In five of the six experiments, the maximum rate of Ca\(^{2+}\) clearance from the cytoplasm occurred at the same time or a few seconds after the maximum rate of rise of free mitochondrial Ca\(^{2+}\) (Fig. 6A, inset). One possible explanation is that the intramitochondrial buffer is increasing as Ca\(^{2+}\) enters the mitochondria. To further test this possibility, we monitored the increase in [Ca\(^{2+}\)]\(_\text{mit}\) following two successive applications of GnRH (Fig. 6B). The first application resulted in a rapid and robust increase in [Ca\(^{2+}\)]\(_\text{mit}\) whereas the second application produced a more gradual increase even though the [Ca\(^{2+}\)]\(_\text{mit}\) oscillations were very similar in both cases. The second application of GnRH was intentionally given before the rhod-2 signal was fully recovered to base line. If full recovery was allowed, the [Ca\(^{2+}\)]\(_\text{mit}\) accumulation would appear similar in both cases as in Fig. 5.

Mitochondria Lower [Ca\(^{2+}\)]\(_\text{cyt}\) near Sites of Secretion—We have shown that mitochondria take up cytoplasmic Ca\(^{2+}\) as measured by the spatially averaged fluorescence of dyes. Does this uptake have significant local physiological effects? We decided to test whether mitochondria influence the Ca\(^{2+}\) dynamics near sites of secretion by using simultaneous measurements of whole cell Ca\(^{2+}\) current, [Ca\(^{2+}\)]\(_\text{cyt}\) and membrane capacitance (\(C_m\)), an indicator of exocytosis. Trains of depolarizing pulses (100 ms, +15 mV) were applied to gonadotropes at 5 Hz for 2 s to elicit periodic Ca\(^{2+}\) entry through voltage-gated Ca\(^{2+}\) channels. They evoked exocytosis. During the train, the membrane capacitance seemed to rise in two components (Fig. 7A); the first few pulses mobilized a small (~20 femtofarad) pool of “immediately releasable” vesicles; then a much larger, second “readily releasable” pool was recruited (32, 33). CCCP dramatically enhanced the release of the readily releasable pool but not of the immediately releasable pool. Furthermore, the capacitance continued to increase throughout each interpulse period in the presence of CCCP, whereas little or no increase was seen in the absence of CCCP during this period (Fig. 7A, compare traces at arrows). Thus, when mitochondrial Ca\(^{2+}\) uptake is impaired by the presence of CCCP, [Ca\(^{2+}\)]\(_\text{cyt}\) apparently remains high enough to cause continued exocytosis, without simultaneous Ca\(^{2+}\) influx. In three experiments, Calcium Green-3N (100 μM) was included in the pipette to monitor [Ca\(^{2+}\)]\(_\text{mit}\). The increase in Calcium Green 3N fluorescence during the 2 s depolarizing train (ΔF) was roughly linear, and the resting fluorescence intensity just prior to the train (F) was essentially constant throughout an experiment. CCCP increased this linear rate of rise of ΔF/F during the 2 s depolarizing train by 89 ± 20% (n = 3) relative to same cell controls (data not shown). These effects are not likely due to changes of ATP levels, because in these whole cell experiments the pipette
Mitochondria Shape Cytoplasmic Calcium Oscillations

solution contained 2 mM ATP and the bath contained oligomycin-B throughout to block mitochondrial ATP hydrolysis. Examination of Fig. 7B shows that CCCP modestly accelerated the progressive reduction in the Ca$^{2+}$ current, an effect that would have, by itself, tended to reduce, rather than increase, the amount of exocytosis during the voltage pulses in CCCP.

**DISCUSSION**

Pituitary gonadotropes are well suited for study of physiologically relevant cellular Ca$^{2+}$ dynamics. They respond robustly to their natural releasing hormone with periodic elevations of [Ca$^{2+}$]$_{cyt}$, the essential stimulus for secretion of gonadotropins. Like skeletal muscle, gonadotropes shuttle Ca$^{2+}$ among various intracellular compartments during their physiological response instead of relying on significant Ca$^{2+}$ fluxes across the plasma membrane (16, 17). We show that mitochondria of gonadotropes have a significant role in this physiological Ca$^{2+}$ response. They take up Ca$^{2+}$ during hormonally induced [Ca$^{2+}$]$_{cyt}$ elevations rapidly enough to account for 40% of the cytosolic Ca$^{2+}$ clearance from the cytosol during each cycle. When [Ca$^{2+}$]$_{cyt}$ is approximately 1 μM, the mitochondrial component of clearance lowers [Ca$^{2+}$]$_{cyt}$ at a rate of 400 nM s$^{-1}$ and limits the amount of exocytosis. Further, the Ca$^{2+}$ binding ratio of the mitochondrial matrix averages 40-fold higher than that of the cytosol, so mitochondria contain powerful Ca$^{2+}$ buffers. Finally, after [Ca$^{2+}$]$_{cyt}$ returns to resting levels, accumulated Ca$^{2+}$ is slowly extruded from the mitochondria, with the principal route being the CGP-37157-sensitive mitochondrial Na$^+$-Ca$^{2+}$ exchanger.

These results are in general agreement with previous work on chromaffin cells where we measured clearance rates (7), mitochondrial Ca$^{2+}$ binding ratios, and the effect of CGP-37157 (8). In chromaffin cells, mitochondria are the dominant clearance mechanism (84% of clearance), but they too reduce [Ca$^{2+}$]$_{cyt}$ at rates near 400 nM s$^{-1}$ when [Ca$^{2+}$]$_{cyt}$ is around 1 μM. Apparently, mitochondria of the two cell types are comparable in their uptake rates and Ca$^{2+}$ binding ratios, but in gonadotropes, uptake by the endoplasmic reticulum (22) is much faster, so that the mitochondrial component is only 40% of the total. In both cells, the mitochondrial Na$^+$-Ca$^{2+}$ exchanger is the principal route for Ca$^{2+}$ extrusion, but the exchanger in gonadotropes must be 4–10-fold less active because their mitochondria retain Ca$^{2+}$ much longer. The present results also are in qualitative agreement with our early experiments on gonadotropes showing that CCCP can stop GnRH-induced oscillations and release Ca$^{2+}$ into the cytosol (19). Very roughly, our results also fit with classical measurements in isolated heart mitochondria. There the maximum values of Ca$^{2+}$ and H$^+$ fluxes are given as 10 and 55 μmol g$^{-1}$ s$^{-1}$, respectively (34, 35), referred to dry protein. With conversion factors summarized by Scarp (36), the Ca$^{2+}$ influx would become 7 mmol l$^{-1}$ s$^{-1}$, referred to cardiac mitochondrial volume. If we take the mitochondrial volume of gonadotrope as 2% of the cell and the cytoplasmic Ca$^{2+}$ binding ratio as 100, this maximal flux of cardiac mitochondria could reduce [Ca$^{2+}$]$_{cyt}$ at a rate of 1.4 μM s$^{-1}$, quite compatible with rates we measure in gonadotropes.

Now consider the mitochondrial depolarization. We view the large inside-negative mitochondrial membrane potential as the result of a steady electric current of proton export working across a very poor conductor, the mitochondrial inner membrane. Calcium uptake is also a current and reduces the membrane potential by momentarily canceling some fraction of the oppositely directed proton current. (As we saw above, these two maximal values are similar.) When Ca$^{2+}$ influx stops, the proton current repolarizes the mitochondrial membrane potential without delay. The Ca$^{2+}$ uptake-induced mitochondrial depolarization that we report (Fig. 4) gives only 14% of the large increase in TMRE fluorescence obtained with strong depolarization by CCCP. Considering that by the Nernst equation a depolarization of only 18 mV would change the TMRE distribution ratio 2-fold, we can argue that this Ca$^{2+}$ uptake depolarizes by only few millivolts. Thus, a Ca$^{2+}$ uptake sufficient to have a significant impact on Ca$^{2+}$ oscillations and exocytosis and large enough to be detected by TMRE makes but a small reduction in the $-150$ to $-180$ mV membrane potential of energized mitochondria.

We have reported here spatially averaged optical measurements. Very likely there are gradients within the cytosol and heterogeneity among mitochondria (37, 38), so some quantitative relationships are not well resolved. For example, the endoplasmic reticulum and mitochondria may have a special spatial relationship that allows more efficient mitochondrial removal of Ca$^{2+}$ released from the endoplasmic reticulum (39). Our lab has shown that the local [Ca$^{2+}$] near sites of exocytosis is higher than the spatially averaged value during GnRH-induced oscillations (18), and here we have shown that mitochondria take up Ca$^{2+}$ near sites of exocytosis. Hence, the mitochondrial uptake we measure may be rapid because it occurs in regions where the [Ca$^{2+}$] is considerably higher than the averages reported by our dyes. Using ideas described in
Mitochondria Shape Cytoplasmic Calcium Oscillations

other cells (33, 40, 41) for explaining our voltage step experiments, the immediately releasable pool of secretory granules may be those docked vesicles that are near to voltage-gated Ca\(^{2+}\) channels, and the readily releasable pool may be granules that are docked farther from the Ca\(^{2+}\) channels. When mitochondria are fully functional (no CCCP), they would limit the diffusional range of Ca\(^{2+}\), so little spreads beyond the immediately releasable pool. However, when CCCP is present, the Ca\(^{2+}\) can spread to the readily releasable pool, and more vesicles are discharged over a longer period of time. It should be noted that voltage step protocol used to induce exocytosis in experiments like those in Fig. 7 might not accurately reflect hormonally induced exocytosis, because the physiological source of Ca\(^{2+}\) is from IP\(_{3}\)-gated stores and not voltage-gated stores. Our attempts to measure the effects of CCCP on GnRH-induced exocytosis were prevented by the large, uncontrollable [Ca\(^{2+}\)]\textsubscript{cyt} oscillations that occur following stimulation. These oscillations result in a large mitochondrial Ca\(^{2+}\) load that is partially released by the addition of CCCP, which then raises [Ca\(^{2+}\)]\textsubscript{cyt}. The voltage protocol used in Fig. 7 allows a short and relatively small addition of a known amount of Ca\(^{2+}\) (from current records) to the system. Here the mitochondria do not take on a large Ca\(^{2+}\) load, and same cell comparisons of changes in capacitance are possible.

One surprising observation was that in experiments like that of Fig. 6A, the rise of [Ca\(^{2+}\)]\textsubscript{mit} leveled off while [Ca\(^{2+}\)]\textsubscript{cyt} was still falling. One possible explanation, which we propose as a working hypothesis, is that the intramitochondrial buffer is dynamic, increasing as more Ca\(^{2+}\) ions are bound. This is supported by experiments like that in Fig. 6B, where the increase in [Ca\(^{2+}\)]\textsubscript{mit} is greatly slowed during a second challenge with GnRH. The increase in intramitochondrial buffering could occur (i) if a chelating anion such as phosphate were transported in while Ca\(^{2+}\) enters, (ii) if insoluble Ca\(^{2+}\) salts formed in the mitochondrial matrix (38, 42), or (iii) if a rise in intramitochondrial pH enhanced the degree of ionization of the mitochondrial buffers. Consistent with a pH dependence for buffering, we observed an initial transient rise of [Ca\(^{2+}\)]\textsubscript{mit} when CCCP was applied (Fig. 6A). We attribute this rise to acidification of the matrix and weakening of their Ca\(^{2+}\) buffers. Whatever the details of local gradients and intramitochondrial buffering, the mitochondrial uptake of Ca\(^{2+}\) suffices to have a significant impact on physiological Ca\(^{2+}\) oscillations and on exocytosis.

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