Differential Gel Electrophoresis and Transgenic Mitochondrial Calcium Reporters Demonstrate Spatiotemporal Filtering in Calcium Control of Mitochondria*

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Mitochondria must adjust both their intracellular location and their metabolism in order to balance their output to the needs of the cell. Here we show by the proteomic technique of time series difference gel electrophoresis that a major result of neuroendocrine stimulation of the Drosophila renal tubule is an extensive remodeling of the mitochondrial matrix. By generating Drosophila that were transgenic for both luminescent and fluorescent mitochondrial calcium reporters, it was shown that mitochondrial calcium tracked the slow (minutes) but not the rapid (< 1 s) changes in cytoplasmic calcium and that this resulted in both increased mitochondrial membrane polarization and elevated cellular ATP levels. The selective V-ATPase inhibitor, bafilomycin, further enhanced ATP levels, suggesting that the apical plasma membrane V-ATPase is a major consumer of ATP. Both the mitochondrial calcium signal and the increase in ATP were abolished by the mitochondrial calcium uniporter blocker Ru360. By using both mitochondrial calcium imaging and the potential sensing dye JC-1, the apical mitochondria of principal cells were found to be selectively responsive to neuropeptide signaling. As the ultimate target is the V-ATPase in the apical plasma membrane, this selective activation of mitochondria is clearly adaptive. The results highlight the dynamic nature and both spatial and temporal heterogeneity of calcium signaling possible in differentiated, organotypic cells and provide a new model for neuroendocrine control of V-ATPase.

Cell signaling is vital for coordination and homeostasis of all organisms. Elucidation of the intracellular mode of action of hormones, neuropeptides, and other soluble modulatory signals is thus of great interest. Although our detailed understanding of cell signaling processes reflects the enormous investment in its study, it would still be useful to obtain a global view of cell signaling targets, rather than an incremental one. Post-genomic technologies are potentially useful in this regard, although both transcriptomics and proteomics have limitations to match their benefit. The transcriptome of a cell will only change in response to nuclear actions of an extracellular messenger, so rapid effects (less than 10s of minutes) are unlikely to be reflected in meaningful changes in the transcriptome. Potentially, therefore, the proteome is more likely to reflect instantaneous changes in the cell. The main problem with this approach is the difficulty in detecting subtle changes between gels; it is not reasonable to suppose that all changes will be drastic.

The standard two-dimensional PAGE technique has been adapted to overcome this obstacle; difference gel electrophoresis (DiGE) permits pairwise comparison between protein samples on the same gel by labeling each protein sample with a distinct fluor (1). To further aid quantification, it is also customary to run a third sample as a standard. Here, DiGE was applied to elucidate the mode of action of a Drosophila neuropeptide, Capa-1, on the Malpighian (renal) tubule. Capa-1 acts through intracellular calcium to generate nitric oxide and therefore cyclic GMP, and the ultimate target of signaling is thought to be an apical plasma membrane proton pump, the V-ATPase (2, 3). As V-ATPase has not been demonstrated to be regulated by protein phosphorylation, it was of interest to study its probable activation at a proteomic level. Although the Drosophila renal tubule is tiny (only 160 cells), it proved possible to obtain good DiGE gels from pooled samples and to perform a time course of action for the Capa-1 peptide. As expected, V-ATPase subunits were found to vary but, surprisingly, so did a range of mitochondrial enzymes. By generating Drosophila that were transgenic for two independent mitochondrial targeted calcium reporters (one luminescent and one fluorescent), mitochondria in the tube principal cell were shown to receive a direct calcium signal from Capa-1, and this increased both mitochondrial potential and cellular ATP levels, functionally validating the novel mode of action for this peptide suggested by the DiGE time course study.

MATERIALS AND METHODS

Drosophila—Flies were raised in bottles on standard Drosophila medium, at 25 °C and 55% relative humidity, on a 12:12 light/dark cycle. The strains used were as follows: Oregon R, wild-type; c42, a GAL4 driver line specific for the principal cells of the tubule main segment (the cell type on which Capa-1 is known to act (4)); UAS-apoaequorinCytoplasmic, flies carrying the apoaequorin luminescent cytoplasmic calcium reporter under control of the UAS promoter (4); UAS-apoaequorinMembrane, flies carrying the apoaequorin luminescent calcium reporter with a mitochondrial targeting signal under control of the UAS promoter, described below; UAS-mitycam, flies carrying a pericam fluorescent calcium reporter targeted to mitochondria under control of the UAS promoter, described below; and vhaSFD:eGFP, a gene trap construct, in which the V-ATPase SFD subunit is translationally fused to enhanced GFP (5). All transgenic lines were viable as homozygous.

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1 The abbreviations used are: DiGE, difference gel electrophoresis; UAS, upstream activating sequence; PBS, phosphate-buffered saline; GFP, green fluorescent protein.
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gotes, thus facilitating crossing to produce flies in which the relevant reporter was targeted to principal cells.

Neuropeptide Stimulation and Malpighian Tubule Secretion Assays—Week-old adult flies were cold-anesthetized and dissected in Schneider’s medium (Invitrogen). Fluid secretion assays were performed with a modified Ramsay assay, as described previously (6). In essence, tubules were prepared as pairs, joined by a common ureter, and transferred to 10-µl drops of Schneider’s medium in paraffin wax-lined 35-mm Petri dishes under paraffin oil (Sigma). One of the tubule pair was led out of the drop and wrapped around a small insect pin, under the oil, therefore stranding the ureter midway between the drop and the pin. Secretion by the tubule that remained in the drop was collected from the ureter every 5 min, and the diameters of the resulting droplets were measured with an eyepiece micrometer. The volume of secretion could then be calculated from the equation for a sphere. Typically, 20 tubules would be run simultaneously, allowing a 10 experimental + 10 control design to be achieved.

When the diuretic neuropeptide Capa-1 (7) (synthesized by Invitrogen) or other compounds were added, they were dissolved in Schneider’s medium at 10× the final concentration required, and 1-µl droplets were fused with the main bathing drops at the times indicated in the figures.

Generation of Mitochondrial Calcium Reporter Transgenic Flies—Drosophila, transgenic for a luminescent mitochondria-targeted calcium reporter, were generated by cloning a commercial mitochondrially targeted Aequorea victoria apoaequorin construct from the mtAEQ/pMT2 vector (Molecular Probes) into the pUAST vector (8). This construct uses the mitochondrial targeting signal from subunit VIII of human cytochrome c oxidase (9), cloned into the pUAST vector (8), and embryos were germ line-transformed, creating several independent transgenic lines.

Drosophila, transgenic for a fluorescent mitochondria-targeted calcium reporter (“mitycam”), were generated from ratiometric pericam (kind gift of Atsushi Miyawaki (11)) by introducing the changes D148T in the enhanced yellow fluorescent protein domain and E67Q in the calmodulin domain. This produced a single wavelength excitation reporter with a greater sensitivity to Ca2+ ($K_\text{d} = 47 \text{nM}$). This was C-terminally fused to the mitochondrial targeting signal from subunit VIII of human cytochrome c oxidase (9), cloned into the pUAST vector (8), and embryos were germ line-transformed, creating several independent transgenic lines.

Proteomics—For proteomics, pools of 500 rapidly dissected tubules were accumulated in 200-µl aliquots of Schneider’s solution in Eppendorf tubes. The diuretic neuropeptide Capa-1 (7) was dissolved in Schneider’s solution and added to samples at a final concentration of $10^{-7} \text{M}$. This concentration provides maximal stimulation of tubule fluid secretion (7). The samples were mixed by gentle inversion and then stopped after timed intervals of 0, 0.25, 0.5, 1, 2, 5, or 10 min by addition of AUT sample buffer (10 mM Tris/HCl (pH 8.5), 8 M urea, and 2% (w/v) amino-sulfobetaine detergent (ASB14)) and rapid homogenization. Four to 10 experimental replicates of each time point were generated. The protein concentration was determined using a Bio-Rad DC detergent-compatible kit.

Two-dimensional DiGE was performed as described previously (12). Protein samples from individual time points (50 µg) were minimally labeled with Cy3 or Cy5 (200 pmol; Amersham Biosciences). A protein pool consisting of all protein samples included in the study was generated for use as an internal standard and was minimally labeled with Cy2. Proteins labeled with Cy2 (pool), Cy3, and Cy5 were mixed and separated by isoelectric focusing using 13-cm IPG DryStrips (pH 4-7) (Amersham Biosciences) according to the manufacturer’s instructions. Proteins were further separated according to molecular weight using SDS-polyacrylamide gels (12%, Etan Dalt Twelve apparatus; Amersham Biosciences). Following electrophoresis, gels were scanned at appropriate wavelengths for Cy2, Cy3, and Cy5 fluorescence using Typhoon™ 9400 (Amersham Biosciences). Gel images were cropped using ImageQuant™ version 5.2 (Amersham Biosciences), and protein expression was quantified using Biological Variation Analysis software module (Amersham Biosciences). The Cy2 internal standard included in all experiments for normalization purposes allowed both intra- and inter-gel analyses. Protein spots showing significant changes ($p < 0.05$) with respect to

FIGURE 1. Time course difference gel electrophoresis. A, typical DIGE gel of Drosophila adult tubule, viewed for Cy3/Cy5 labels. B, gel marked with picks for mass spectrometry. C, significant changes from DeCyder. Each point represents the volume of corresponding spots on single gels; the lines represent median abundance. The x axis denotes times in seconds from the addition of Capa-1. The genes are those listed in Table 1; note that some genes (left columns) are represented by multiple (adjacent) traces.

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FIGURE 1—continued

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Transport

Spot 354 - CG3762 (vha58-2)
V-ATPase subunit

Spot 642 - CG6048 (vha44)
V-ATPase subunit

Signaling

Spot 1010 - CG17879
14-3-3-c

Spot 1031 - CG17870
14-3-3-c

Mitochondrial

Spot 483 - CG2612 (bhw)
ATP synthase α subunit

Spot 490 - CG2612 (bhw)
ATP synthase α subunit

Spot 494 - CG11154 (ATPsyn b)
ATP synthase β subunit

Spot 500 - CG11154 (ATPsyn b)
ATP synthase β subunit

Spot 1023 - CG9647 (pord)
Porin

Spot 1018 - CG9647 (pord)
Porin

Spot 273 - CG244 (Acon)
Aconitase

Spot 873 - CG9814
Enoyl-CoA hydratase

Spot 558 - CG17604 (Eno)
Enolase

Spot 1020 - CG17604 (Eno)
Enolase

Spot 1119 - CG7113 (acul)
3-
Hydroxyacyl-CoA dehydrogenase

Spot 1129 - CG7834
Electron transport flavoprotein

Spot 453 - CG4347 (UGP)
UTP-G-3-P uroxy/transferase

FIGURE 1—continued
control were picked from a colloidal Coomassie-stained gel and identified by liquid chromatography/tandem mass spectrometry.

Following gel-to-gel matching of spots, statistical analysis (analysis of variance) of normalized protein abundance changes between samples was performed by using the using Biological Variation Analysis software module as described (12).

Proteins within the gel-excised spots were first reduced, carboxy-amido-methylated, and then digested to peptides using trypsin on a MassPrepStation (Micromass, Manchester, UK). The resulting peptides were applied to liquid chromatography/tandem mass spectrometry. The liquid chromatographic separation was achieved with a PepMap C18, 180 μm inner diameter, 15-cm column (LC Packings, Amsterdam, Netherlands). The mass spectrometer was a QToF (Micromass). Fragmentation data were used to search the National Center for Biotechnology Information data base using the MASCOT search engine. Probability-based MASCOT scores were used to evaluate identifications. Only matches with $p < 0.05$ for random occurrence were considered significant (further explanation of MASCOT scores can be found online).

Manual sequence assignment was assisted using the peptide sequencing feature of BioLynx (Micromass).

Dynamic Imaging of V-ATPase Localization—Adult flies were dissected in Schneider’s Drosophila medium (Invitrogen). For vital imaging of V-ATPase expression with the vhaSFD::GFP gene trap line (5), both before (left) and after (right) application of Capa-1 to $10^{-7}$ m. Tubules were counterstained with anti-actin (red) and nuclei were labeled with DAPI (blue) to aid discrimination of apical-basal domains. Arrows show the basal surface of the tubule both pre- and post-stimulation.

| Spot | Gene | Description |
|------|------|-------------|
| 354  | vha68-2 | V-ATPase A subunit |
| 642  | vha44 | V-ATPase C subunit |

Mitochondrial enzymes

| 273  | Acon | Aconitase (mitochondria) |
| 453  | UGP | UTP-glucose-1-phosphate uridylyltransferase |
| 483  | Blw | Bellwether, proton ATP synthase α subunit (mitochondria) |
| 478  | Blw | Bellwether, proton ATP synthase α subunit (mitochondria) |
| 490  | Blw | Bellwether, proton ATP synthase α subunit (mitochondria) |
| 494  | ATPsymb | ATPsymb-β, ATP synthase β subunit |
| 500  | ATPsymb-β | ATPsymb-β, ATP synthase β subunit |
| 873  | CG9914 | Enoyl-CoA hydratase mitochondria |
| 1020 | CG6543 | Short-chain enoyl-CoA hydratase activity EC 4.2.1.17 (fatty acid β-oxidation mitochondrial matrix) |
| 558  | eno | Enolase (glycolytic) |
| 1023 | CG6647 | Porin |
| 1018 | CG6647 | Porin |
| 1029 | CG7834 | Electron transfer flavoprotein complex (sensu Eukarya) |
| 1119 | scully | 7β-Hydroxysteroid dehydrogenase (NADP+) activity (EC 1.1.1.201) |

Cell signaling

| 1031 | 14-3-3ζ | Tryptophan hydroxylase activator activity; protein kinase C inhibitor activity |
| 1010 | 14-3-3ζ | Tryptophan hydroxylase activator activity; protein kinase C inhibitor activity |

In groups of 20 in Schneider’s medium. They were incubated in the dark with coelenterazine (the cofactor for apoaeequorin) for 2–4 h. Samples were then placed in a Berthold luminometer, and base-line luminescence was measured for at least 30 s before automated injection of Capa-1 (7) to a final concentration of $10^{-7}$ m. Real time readings then continued for at least 3 min, with sampling every 0.1 s. Quantification was as for cytoplasmic aequorin; at the end of the experiment, total tissue luminescence was discharged by incubating in a calcium-containing buffer containing Triton X-100. This allowed the calculation of calcium concentration by back-integration using a routine written in Perl (4).
Mitycam is an inverse pericam, with $K_d = 47 \text{nM}$. It is thus ideally suited for detecting small changes in mitochondrial calcium. This peri-cam has a single excitation wavelength peak at ~485 nm, and therefore ratiometric quantification is not possible.

Real time images of the tubule-expressing mitycam were captured, and average fluorescence values for specified regions of interest were calculated for each frame. As mitycam is an inverse reporter, these values were subtracted from a higher arbitrary value to give fluorescence decrease and thus an estimate of calcium increase over time.

![Images of mitycam localization](image)

**FIGURE 3.** Capa-1 signaling elevates mitochondrial calcium. A and B, localization of mitycam (mitochondrially targeted pericam) within Malpighian tubule principal cells: A, transverse section, and B, longitudinal section. Mitochondria are seen throughout the cell, but particularly within the apical microvilli the striations are apparent, corresponding to individual mitochondria. C-E, mitycam co-localizes with Mitotracker dye (Molecular Probes and Invitrogen). C, mitycam channel (green); D, Mitotracker channel (red); E, co-localization. Although there is some additional transport of Mitotracker dye into vesicles, co-localization is evident. F, comparison of cytoplasmic (gray) and mitochondrial (black) responses to calcium upon Capa-1 stimulation (arrow). Typical experiment: traces are from tubule samples from two different groups of adult Drosophila, one transgenic for UAS-apoaequorin$_{cytoplasm}$, and the other transgenic for UAS-apoaequorin$_{mt}$, and both are driven by GAL4 line c42, which drives expression to principal cells of tubule. G, summary of calcium changes induced by Capa-1. Bars represent the increases in the following: [Ca$^{2+}$], first the initial peak (~1 s) of cytoplasmic calcium; [Ca$^{2+}$], second the secondary peak (100–200 s) of cytoplasmic calcium; and [Ca$^{2+}$]$_{mt}$, the sustained rise (100–200 s) in mitochondrial calcium. Data are presented as mean ± S.E., n = 13. H, confocal imaging of the mitochondrial calcium reporter mitycam, under UAS control and driven by c42 (principal cell specific (40)) GAL4 driver. I, effect of Capa-1 stimulation on the mitochondria in the single cell outlined in yellow in H. Although the calcium measurement technologies differ fundamentally between F and I, note the congruence in the signal reported by each. Scale bars represent 10 $\mu$m.
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coupled to an inverted Zeiss microscope. Tubules were imaged in real time with settings normally employed for GFP and dsRED, as these are close to the green and metachromatic red emissions, respectively, of JC-1. Pilot experiments had shown that uptake of JC-1 was saturated within 10 min. This rapid uptake of the dye, which facilitated our experiments, is probably a result of active uptake via an organic solute transporter. These are conspicuously abundant in the tubule transcriptome (14, 15).

**RESULTS**

**Proteomics**—The two-dimensional proteome of adult *Drosophila* tubule is relatively simple, stable, and clearly defined (Fig. 1, A and B). The effect of application of the diuretic Capa-1 peptide was assessed by a DiGE time course on matched pairs of neuropeptide/vehicle-only treated batches of tubules. The choice of time points (0, 0.5, 1, 2, 5, and 10 min) was informed by the known time course of action of Capa-1 peptides, which produce a stable increase in secretion and elevation of transepithelial potential, 5–10 min after application (7, 16). At this interval, it is unlikely that transcriptional changes can significantly affect tubule function, and so a proteomic approach is indicated.

Gel DeCyder software identified 18 spots that varied significantly over the time course of the experiment, as assessed by analysis of variance (Fig. 1C). A few spots showed a monotonic increase or decrease; most showed a complex variation. However, multiple spots corresponding to the same proteins were identified, increasing our confidence in their identification as genuinely varying proteins. The hits (see Table 1) fell into three categories as follows: V-ATPase subunits, mitochondrial enzymes, and a cell signaling protein. The V-ATPase is a complex holoenzyme, which in *Drosophila* is formed of 13 subunits encoded by 34 genes (15); the hits are to subunits that are believed to form the plasma membrane form of the pump (15), specifically the V1 cytoplasmic head group. This is not surprising, as the V1 subunits are intensely hydrophobic integral membrane proteins, and so would not run into a two-dimensional gel. Similarly, the mitochondrial proteins are all matrix, and thus soluble, proteins. The hits are to both metabolic enzymes (such as enolase or dehydrogenases) and to two subunits of the F-type ATP synthase head group. In one case, i.e. porin, two neighboring spots (1018 and 1023) showed reciprocal changes, implying a shift between two states, possibly reflecting phosphorylation.

**ATP Assays**—For each sample, 20 tubules were dissected and placed into 25 μl of Schneider’s medium. Samples were either left untreated (controls) or treated with Capa-1 for 10 min (10^{-7} M) and/or the V-ATPase inhibitor bafilomycin for 10 min (10^{-6} M). For Capa-1 + bafilomycin samples, tubules were pre-treated with bafilomycin for 10 min, prior to a 10-min stimulation with Capa-1. Triplicate samples were generated for each condition. Control samples were left for 20 min.

All samples were quenched with 25 μl of ice-cold 10% (v/v) perchloric acid. Samples were sonicated and spun at 5,000 × g for 1 min. Supernatants were removed, and 1 μl of Universal Indicator (BDH) was added to each sample. Samples were neutralized with 1.5 M KOH, 60 mM HEPES. Precipitates were spun down, and 50 μl of supernatant from each sample was used for ATP measurements using a luciferin-based ATP determination kit (Invitrogen). The assay was conducted using a Berthold tube luminescence according to manufacturer’s instructions using ATP standards at final concentrations between 1 nM and 1 μM. Tubule samples were used at 1:9 dilution (v/v) in a final volume of 200 μl and were assayed in duplicate. The ATP concentrations of tubule samples were calculated from the standard curve. Results are expressed as picomoles of ATP ± S.E. (n = 6).

In *Drosophila*, a variety of genetic resources allow the data to be extended from proteomics back to the organism. A GFP-tagged subunit of the V-ATPase SfD subunit (5), which forms part of the V1 head group, is apically localized in *Drosophila* epithelia and allows organo-
typic monitoring of V-ATPase positioning within the cell. Confocal sections (Fig. 2) show the majority of vhaSFD to be apically located both before and after Capa-1 stimulation, reflecting the relatively high basal secretion levels by tubule. However, the resting tubule also shows some basolateral V-ATPase, which vanishes after Capa-1 stimulation. We thus conclude that Capa-1 plays only a modest role in remodeling the apical V-ATPase by release of any available basolaterally anchored V-ATPase head groups. So mobilization of V-ATPase subunits, apical V-ATPase by release of any available basolaterally anchored groups, is thus concluded that Capa-1 plays only a modest role in remodeling the cell. Confocal sections (Fig. 2) show the majority of vhaSFD to be apically located both before and after Capa-1 stimulation, reflecting the relatively high basal secretion levels by tubule. However, the resting tubule also shows some basolateral V-ATPase, which vanishes after Capa-1 stimulation. We thus conclude that Capa-1 plays only a modest role in remodeling the apical V-ATPase by release of any available basolaterally anchored V-ATPase head groups. So mobilization of V-ATPase subunits, although a known method of controlling V-ATPase subunits, is unlikely to explain the full response to Capa-1.

Real Time Measurement of Mitochondrial Calcium—How can the completely unexpected change in mitochondrial enzymes be explained? Capa-1 is known to signal through intracellular calcium and nitric oxide, which are known to activate or inhibit mitochondrial respiration, respectively (22–25). Pyruvate dehydrogenase in particular is activated by mitochondrial calcium (26–28), although the number of mitochondrial matrix enzymes identified here (Table 1) implies a major remodeling of the mitochondrial matrix, including the cytoplasmic head groups of the F-type ATP synthase. As mitochondrial calcium ([Ca$^{2+}$]$_{mit}$) can respond to changes in cytoplasmic calcium (27), the differential responses of cytoplasmic and mitochondrially targeted apoaequorin transgenes, targeted to principal cells with the c42 GAL4 driver, were followed. As reported previously (4, 7), Capa-1 induces a very rapid cytoplasmic calcium transient within the 1st second and then a slower, sustained rise that peaks in 100–200 s and can last for many minutes (Fig 3F). By contrast, mitochondrial calcium levels reported by aequorin are temporally filtered; the calcium is steady over the first few seconds but clearly follows the slower peak of cytoplasmic calcium.

We also generated flies transgenic for UAS-targeted mitochondrial pericam. This is an entirely different calcium measurement technology, using the fluorescent pericam reporter (11), and permits imaging of single cells. The Capa-1 response observed is similar to that observed using the aequorin technology, with a slow increase that peaks ~100 s after stimulation. Recordings from single principal cells report the same kinetics (Fig. 3, H and I). It is thus clear that mitochondria receive the slow, but not the fast, calcium signal associated with Capa-1 stimulation. This corresponds with the spatial distribution of calcium signaling within the cell; the rapid transient is thought to be localized to the basolateral side of the cell, whereas the sustained rise involves the whole cell. Most of the mitochondria are physically located within the apical microvilli of the tubule (29) (see Fig. 3, A–E). There is thus a natural diffusion barrier that would preclude most of the principal cell mitochondria from "seeing" rapid calcium transients.

Apical Mitochondria Respond Selectively to Capa-1 Signaling—To test this idea, fluorescence changes in smaller regions of interest, corresponding to just the apical or basolateral mitochondria, were compared with the responses of the whole cell in mitycam transgenic tubules. The results of a typical experiment are shown in Fig. 4; the whole cell mitochondrial calcium response is clearly seen to be an average of a near-constant basolateral signal and a much more dynamic apical signal. The calcium signal to mitochondria is thus spatially, as well as temporally, filtered.

Mitochondrial Calcium Signal Activates Mitochondria and Increases Cellular ATP—Clearly, mitochondria are receiving a filtered calcium signal from the cytoplasm. However, is this sufficient to produce a real change in mitochondrial output? Activated mitochondria show a hyperpolarization of the inner mitochondrial membrane; and under favorable circumstances, this can be imaged directly with potential sensitive dyes such as JC-1 (30, 31). This dye labels quiescent mitochondria green but forms red "J-aggregates" in the membrane of activated mitochondria. When tubules are incubated in JC-1, and imaged by real time confocal microscopy, there is a clear increase in fluorescence in the red channel (Fig. 5A) and with a time course consistent with the calcium signal (cf.
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Fig. 3). Interestingly, although both basal and apical mitochondria respond to Capa-1, the basal mitochondria are highly active even under basal conditions; the biggest change in fluorescence (and thus mitochondrial activity) is thus seen in the apical mitochondria (Fig. 5A) located in the apical microvilli. There is thus spatial, as well as temporal, heterogeneity in mitochondrial response within a single cell.

The final demonstration of the physiological relevance of the mitochondrial calcium signal would be that the changes identified by DiGE and JC-1 imaging actually increase the availability of ATP to the cell. Accordingly, we assayed ATP in tubules directly. Because the apical V-ATPase is thought to energize tubule transport, the assays were repeated in the presence of bafilomycin, a selective V-ATPase inhibitor that acts potently on tubules (32, 33). The results (Fig. 5B) are interesting from two viewpoints. First, Capa-1 increases tubule ATP levels significantly as predicted. Second, bafilomycin also increases tubule ATP, presumably by inactivating a major ATP-utilizing enzyme, and so confirming the major role of V-ATPase in this tissue.

By what mechanism is mitochondrial calcium elevated? By analogy with other systems, the mitochondrial calcium uniporter is the likeliest route. Ru360 (the active compound in ruthenium red) is a potent and selective blocker of this uniporter (34). The mitochondrial calcium response of Drosophila tubules to Capa-1, pre-exposed to 20 μM Ru360 for 1 h, is completely abolished (Fig. 5C). Ru360 also blocks the increase in cellular ATP elicited by Capa-1 (Fig. 5D). We thus conclude that the effect of Capa-1 in increasing ATP levels depends obligatorily on a mitochondrial calcium signal transduced through a Ru360-sensitive mechanism.

Concordance of Different Measures of Capa-1 Action—This study has revealed new dimensions to neuropeptide action, and with better temporal resolution than available previously. Is it possible to compare these time courses, to see whether they are consistent with asserting a causal effect of mitochondrial calcium? Fig. 6 shows such an attempt. Although the temporal resolution of fluid secretion assays is poor (normally 10 min), we were able to perform an experiment with 5 min of resolution (Fig. 6A). Although there is some increase in secretion 0–5 min after Capa-1 addition, this shows that the main increase in secretion is 5–10 min after addition. This increase persists for some time (10s of minutes). The cytoplasmic calcium response (after the initial transient) peaks after 100–200 s and approaches baseline over the next 20 min (Fig. 6). Mitochondrial calcium follows the slower peak closely and is quite early enough to mediate most of the diuretic action of Capa-1 (Fig. 6C). The proteomic traces are highly variable in nature and so are represented here by just one; however, Fig. 1C also shows that several traces show a reduction at 30 s, followed by an increase at 2 min, and the largest increase at 600 s. This latter increase maps well to the acceleration of fluid secretion in Fig. 6A and is consistent with mitochondrial calcium triggering long term changes, perhaps through phosphorylation, as has been suggested recently (35), that persist long after mitochondrial calcium returns to base line.

DISCUSSION

This proteomically led approach has provided some intriguing insights into the action of the Capa-1 neuropeptide. It is clear that there are two distinct signaling branches, the mobilization of V-ATPase subunits to the apical surface and the activation of apical mitochondria. Which is more important? In view of the only modest redistribution of V-ATPase we were able to show (Fig. 2), it is the direct action on apical mitochondria (Figs. 3–5), and hence the ATP supply, which is likely to be the most important. Mitochondrial regulation would provide an elegant and potent control of the highly energy-demanding V-ATPase transport, which is physically intimately close to the apical mitochondria, on the microvillar membrane (Fig. 7). This dual signaling pathway would also explain the relatively slow response of the tubule to this neuropeptide; whereas the response to leucokinin (a neuropeptide that targets the chloride shunt conductance) is effectively complete within 1.5 s (36), the response to Capa-1 builds slowly over around 10 min (7).

By what mechanism is calcium acting? Because the time course of capa action is too rapid to for either changes in transcription or protein synthesis to have a major impact, the proteomic results imply a major remodeling of the mitochondrial matrix, affecting the solubility of multiple enzymes (both metabolic, and the ATP synthase (Table 1). We speculate that calcium may affect the tethering of these enzymes or their phosphorylation state, as recently shown for mitochondria given a sufficient calcium signal to induce apoptosis (35), or even induce a gel-sol transition, as it does in eukaryotic cytoplasm (37).
It is interesting to note that all the mitochondrial assays shown here (proteomic, calcium, membrane polarization, and ATP generation) all show a time course that agrees well with that of the increases in membrane potential (16) and fluid production (7) as reported previously (Fig. 6). Although an increase in lumen-positive potential is generally taken as evidence of up-regulation of V-ATPase, our data have produced an entirely new model for regulation of V-ATPase by modulation of mitochondrial function, and thus of ATP supply.

Why are basal mitochondria constitutively active (Fig. 4)? The classical view is that insect epithelia are energized by the apical V-ATPase (38), and so the apical membrane dominates current thinking in insect biology. However, transepithelial transport requires movement across two membranes; and a recent microarray data set for tubules has demonstrated extraordinary abundance of mRNAs for multiple organic solute transporters (15). In addition, the basolateral Na$^+$/K$^+$-ATPase is at least as abundant at the mRNA level as the apical V-ATPase (15) and may play a much more important role than previously suspected in cellular homeostasis (39). It is thus entirely plausible that the basolateral membrane is constitutively active, requiring high levels of ATP, whereas the apical membrane is regulated by neuropeptides via control of ATP supply.

Given that there is spatial heterogeneity in mitochondrial function, then how are the mitochondria controlled separately? The key may be in the structure of the tubule. Although the basolateral mitochondria are intimately associated with the plasma membrane, the majority of their surfaces is in contact with the cytoplasm (29). By contrast, most of the apical mitochondria are inserted directly into the microvilli, insulating them from transient changes in cytoplasmic calcium (Fig. 3).

By drawing attention to mitochondria, these results have also raised yet another possible modality of Capa-1 signaling, i.e. an action of nitric oxide on mitochondria by competitive inhibition of cytochrome oxidase (23, 25). However, our data downplay the possibility, as Capa-1 signaling demonstrably activates mitochondria (Fig. 5), whereas nitric oxide would inhibit them. However, this does not exclude the possibility that NO may ultimately act to limit Capa-1 signaling, and we were not able to detect such an effect over the time course of our experiments.

We have reported previously the use of a cytoplasmic apoaequorin transgene, under UAS control, which allows real time measurement of intracellular calcium by luminometry. In this study, two such resources are described as follows: mitochondrially targeted apoaequorin, which provides for quantitative real time luminometry; and mitochondrially targeted pericam, which is harder to quantify but ultimately allows for fluorescence imaging of calcium changes. To our knowledge, this is the first time that animals have been made transgenic for mitochondrial calcium reporters, and so these lines constitute valuable resources for the monitoring of calcium in an organotypic context. These results also show the importance of doing so; the polarization of the principal cell and the accumulation of mitochondria at subcellular sites of intense metabolism (like the apical microvilli) would be hard to reproduce in cell lines.

In general, this use of DiGE (1) in monitoring the time course of neuropeptide action may be of great value in elucidating the mode of action of messengers, as it has the potential (as shown here) to reveal unexpected results, as well as confirm predicted results. For those organisms where the genome (and hence the computed proteome) is relatively well defined, such DiGE studies may be a potent tool for post-genomic endocrinology.

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