Selective Transfer of Calcium from an Acidic Compartment to the Mitochondrin of Trypanosoma brucei

MEASUREMENTS WITH TARGETED AEQUORINS*

Organelle compartments are used by cells as reservoirs of exchangeable Ca$^{2+}$ and as Ca$^{2+}$ buffers. The following study uses recombinant aequorins (CYT-AEQ and MT-AEQ) to measure the dynamics of Ca$^{2+}$ flux between organelles in procyclic forms of the pathogenic protozoan, Trypanosoma brucei. Emphasis is placed on the exchange between an acidic Ca$^{2+}$ reservoir and the mitochondrion. The mammalian mitochondrial targeting sequence was functional in trypanosomes as determined by immunoblots, immunolocalizations, and the ing sequence was functional in trypanosomes as determined by immunoblots, immunolocalizations, and the observation that MT-AEQ was in a compartment whose maintenance of Ca$^{2+}$ stasis by inducing Ca$^{2+}$ influx across the plasma membrane. [Ca$^{2+}$]_{cyt} became slightly elevated to 410 ± 100 nM, whereas [Ca$^{2+}$]_{mit} became selectively increased approximately 12-fold, with a broad peak at 4.8 ± 1.9 µM. At the peak, the mitochondrion contained approximately three times more free Ca$^{2+}$ than the cytosol. However, mitochondrial retention of the Ca$^{2+}$ was transient. Similar selective transport into the mitochondrion was observed when Ca$^{2+}$ efflux from an acidic compartment was induced with monensin (2 µg/ml) in the presence of 5 mM EGTA. [Ca$^{2+}$]_{cyt} was transiently elevated to 400 ± 50 nM, whereas [Ca$^{2+}$]_{mit} was elevated to 3.5±1.3 µM. When cells were treated sequentially with monensin (2 µg/ml) and then melittin (200 nM), mitochondrial Ca$^{2+}$ transport was normal. However, [Ca$^{2+}$]_{lyt} became elevated to a level that was 1.4-fold higher than with melittin alone. Overall, these data demonstrate that the trypanosome mitochondrin is not a reservoir of exchangeable Ca$^{2+}$ in the resting cell. However, Ca$^{2+}$ is selectively channeled to the mitochondrion from the plasma membrane or acidic Ca$^{2+}$ storage compartment. Additionally, the acidic compartment contributes to maintenance of Ca$^{2+}$ homeostasis in response to melittin.

Cell survival is dependent upon the ability to receive signals from the environment and initiate appropriate changes in cell activity. This property of cells is especially important in parasitic organisms with digenetic life cycles where multiple environments are encountered and the environment may contain mechanisms to actively seek and destroy the intruder. Trypanosomes of the brueei group are flagellated protozoa that produce lethal infections in humans and livestock throughout sub-Saharan Africa. During the course of the trypanosome infection, the invading cells must adapt to life in the mammalian and insect hosts, as well as to changing environments within these hosts. We are interested in the signal mechanisms within Trypanosoma brucei that allow them to coordinate life cycle events. Emphasis is placed on Ca$^{2+}$ pathways because Ca$^{2+}$ can function as a pluripotent regulatory molecule. Conversely, prolonged exposure to elevated [Ca$^{2+}$]_{lyt} can result in cell death (1, 2). Trypanosome survival therefore depends upon redundant energy-dependent processes that control cytosolic free Ca$^{2+}$ concentrations ([Ca$^{2+}$]_{cyt}) (3–8). The present study examines within intact trypanosomes the relative importance of several Ca$^{2+}$-transporting organelles.

African trypanosomes exhibit some unusual features when considering organelle Ca$^{2+}$ transport. In the present report, emphasis is placed on in vivo measurement of Ca$^{2+}$ transport by the mitochondrin and acidic Ca$^{2+}$ storage compartment. In mammalian cells, the mitochondrin is used as a Ca$^{2+}$ buffer to protect against large changes in [Ca$^{2+}$]_{lyt} (9). Typically, isolated mammalian mitochondria can lower medium Ca$^{2+}$ to a set point of approximately 700 nM (10). However, recent studies with targeted aequorins demonstrate that mammalian mitochondria selectively receive Ca$^{2+}$ from other organelles perhaps by sensing microdomains of elevated Ca$^{2+}$ in the vicinity of the organelle (11–15). By contrast with mammalian cells, T. brucei are primitive organisms, and sequence comparisons suggest that their lineage represents the oldest branch of eukaryotic cells that contain a mitochondrin (16). The mitochondrin of T. brucei contains several novel features, including catedulated maxicircle and minicircle DNA, and a requirement for RNA editing to express mitochondrin-encoded genes (17, 18). During the trypanosome life cycle, the mitochondrin undergoes extensive developmental changes (19). In the mammalian host, the mitochondrin lacks cytosomes and Krebs cycle enzymes (19). Instead, the mitochondrin contributes to NADH oxidation by means of an alternative oxidase (20). A membrane potential is maintained in bloodstream forms at the expense of

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ATP (21) or through activity of the alternative oxidase (22). By contrast, the procylic or insect form of the trypanosome contains a large reticulized mitochondrion that extends throughout the length of the organism. In procylic forms a mitochondrial membrane potential is maintained by a complete respiratory chain (19). Permeabilized cells have been used to demonstrate that mitochondria from both life cycle stages can accumulate large quantities of Ca$^{2+}$ with a set point around 700 nM (4). However, the relative importance of the trypanosome mitochondrion as a reservoir of stored Ca$^{2+}$ has not been adequately explored in either life cycle stage. Yet it has been proposed that Ca$^{2+}$ release from the trypanosome mitochondrion might alter [Ca$^{2+}$]$_{cyt}$ when resting cells are treated with thapsigargin (23) or nigericin (23). These conjectures are only true if the level of Ca$^{2+}$ in the resting mitochondrion is high enough to affect cytoplasmic Ca$^{2+}$ levels.

In addition to the mitochondrion, we have described an acidic compartment that is nonmitochondrial in nature (7). We showed that this compartment released Ca$^{2+}$ in response to the K$^+$/H$^+$ antiporter, nigericin. Enough Ca$^{2+}$ was released to elevate [Ca$^{2+}$]$_{cyt}$ by 3-fold above the resting level (7), suggesting that large quantities of exchangeable Ca$^{2+}$ were stored in this compartment. Later it was shown that the Na$^+$/H$^+$ antiporter, monensin, also released Ca$^{2+}$ from the acidic compartment (24). Ca$^{2+}$ transport into the acidic compartment required a vanadate-sensitive Ca$^{2+}$-ATPase (8), and Ca$^{2+}$ release occurred via a Ca$^{2+}$/H$^+$ exchanger when the organelle pH gradient was collapsed (24). This organelle has also been referred to as the acidocalcisome (8). To date, no evidence exists demonstrating that T. brucei acidocalcisome is a separate or novel organelle. In mammalian cells, acidic Ca$^{2+}$ pools have been described (25, 26), and it is presumed that these pools reside within the lysosome or endocytic vesicles. Histochemical and x-ray microprobe analysis of T. brucei organelles demonstrate that large amounts of Ca$^{2+}$ are stored in a compartment that may be lysosomal in origin (27). Whether the acidic compartment contributes to Ca$^{2+}$ homeostasis in situ is not known. Moreover, the fate of Ca$^{2+}$ released from this compartment is also not known.

In the present study, interactions between the mitochondrion and acidic Ca$^{2+}$ storage compartment are investigated. Melittin is used to initiate Ca$^{2+}$ influx across the plasma membrane (28), whereas monensin is used to disrupt the acidic Ca$^{2+}$ storage compartment (24). Organelle Ca$^{2+}$ transport is measured during the return to homeostasis. To measure organelle Ca$^{2+}$ transport in vivo, the Ca$^{2+}$-sensitive photoprotein aequorin has been targeted to the mitochondrial matrix space or expressed without a localization signal to accumulate in the cytoplasm. When reconstituted in vivo with coelenterazine, recombinant aequorins have recently been used to monitor free Ca$^{2+}$ concentrations in the nucleus (29–32), cytoplasm (33), mitochondria (11–15), and ER (34) of a wide range of mammalian cells. We have previously used targeted aequorins to quantify Ca$^{2+}$ content of the trypanosome nucleus (35). In the present study, targeted aequorins are used to quantify [Ca$^{2+}$]$_{mit}$ in resting and stimulated cells. We report that the mitochondrion does not function as a reservoir of exchangeable Ca$^{2+}$ in the resting cell. However, during the signaling process, the majority of Ca$^{2+}$ that enters the cell from across the plasma membrane or is released from the acidic compartment selectively enters the mitochondrion. The mitochondrion retains this Ca$^{2+}$ transiently. When the mitochondrion releases this sequestered Ca$^{2+}$, other homeostatic organelles must return [Ca$^{2+}$]$_{cyt}$ to the resting level. None of these results were predicted from studies with permeabilized cells.
were immunized with GST-aequorin fusion protein reconstituted to a final concentration of 1 mg/ml in Ribi's adjuvant. For immunolocalization, rat antisem was affinity purified by the method of Olmstead (39) using recombinant GST-aequorin as the immunosorbent. Then, the antibodies were preabsorbed with normal porcine cells that had been fixed for 10 min with 3% paraformaldehyde and permeabilized for 10 min with 0.1% Triton X-100. The procytoxic and the attached antibodies were centrifuged at 10,000 x g for 5 min and discarded. Antibodies in the supernatant were further preabsorbed with a 50-fold excess of recombinant GST. The purified anti-aequorin antibodies were stored in 0.92% sodium azide and 0.1% bovine serum albumin at –20 °C.

Luminescence—Transformed cells at a density of 1.5–2 x 10⁶ cells/ml were incubated with 2.5 μM coelenterazine for 3 h in SDM79 and 15% fetal bovine serum. The cells were then pelleted and resuspended at the same density in reaction buffer (116 mM NaCl, 5.4 mM KCl, 0.8 mM MgSO₄, 5.5 mM glucose and 50 mM Hepes, pH 7.0) to remove the excess coelenterazine. Photon pulses from the Ca²⁺–dependent oxidation of coelenterazine were detected with a home built lumimometer. The lumimometer consisted of a stirred room temperature macrophotometer located 7 cm from the unfiltered opening to a Hamamatsu R1572P low dark current photomultiplier tube. Mirrors around sample compartment directed light to the photomultiplier tube opening. Output from the photomultiplier tube was collated with a photon counting board (Photom Technology International) and processed with software v.2600 from the Delta Scan dual wavelength fluorimeter (Photon Technology International). The luminescence was monitored at the rate of five data points/s. The cells were lysed with 0.1% Triton X-100 and 10 mM CaCl₂ at the end of every experiment to discharge the remaining aequorin. The luminescence detected after that time was considered background. To calculate the Ca²⁺ concentration from the aequorin luminescence, raw data were smoothed, and the background was subtracted from every data point. The data were saved as ASCII files and were processed off-line by a Fortran program in which the kinetic equation [Ca²⁺] = [10⁻³⁺⁻⁺⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻‥⁻‥⁻‥⁻‥⁻‥⁻‥⁻‥⁻‥⁻‥⁻‥⁻‥⁻‥⁻‥⁻‥⁻‥⁻‥⁻‥⁻‥⁻‥⁻‥⁻‥⁻‥⁻‥⁻‥⁻‥⁻‥⁻‥⁻‥⁻‥⁻‥⁻‥⁻‥⁻‥⁻‥⁻‥⁻‥⁻‥⁻‥⁻‥⁻‥⁻‥⁻‥⁻‥⁻‥⁻‥⁻‥⁻‥⁻‥⁻‥⁻‥⁻‥⁻‥⁻‥⁻‥⁻‥⁻‥⁻‥⁻‥⁻‥⁻‥⁻‥⁻‥⁻‥⁻‥⁻‥⁻‥⁻‥⁻‥⁻‥⁻‥⁻‥⁻‥⁻‥⁻‥⁻‥⁻‥⁻‥⁻‥⁻‥⁻‥‥⁻‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥‥…..
nM melittin, the pCYT-AEQ transformants show an immediate spike (possibly an injection artifact), followed by a transient elevation from the resting level of Ca\(^{2+}\) to 1.2 ± 0.4 \(\mu M\) (n = 16) (Fig. 3C). Homeostatic pathways decreased [Ca\(^{2+}\)\(_{\text{cyt}}\) over a 60-s period to 570 ± 160 nM (n = 16). The same dose of melittin caused a similar injection spike in [Ca\(^{2+}\)\(_{\text{mit}}\]) (Fig. 3D). The [Ca\(^{2+}\)\(_{\text{mit}}\)] was then rapidly increased up to 8.4 ± 2.7 \(\mu M\) (n = 13), which was approximately 7-fold higher than the peak value of [Ca\(^{2+}\)\(_{\text{cyt}}\)]. A new steady level of [Ca\(^{2+}\)\(_{\text{mit}}\)] at 1.50 ± 0.5 \(\mu M\) (n = 11) was reached. The elevation in [Ca\(^{2+}\)\(_{\text{mit}}\)] was not the result of a direct interaction between melittin and the mitochondrion. The calcium channel blocker lanthanum completely inhibited Ca\(^{2+}\) influx into the cytosol (dashed curve in Fig. 3C).

Under these conditions, melittin was without effect on [Ca\(^{2+}\)\(_{\text{mit}}\)] (dashed curve in Fig. 3D), showing that the rise in [Ca\(^{2+}\)\(_{\text{mit}}\)] was dependent upon Ca\(^{2+}\) influx across the plasma membrane. Finally, Fig. 3 (E and F) shows that the luminescence of MT-AEQ recombinant protein originated from the mitochondrial matrix. When the mitochondrial electrophoresis potential was disrupted with the respiratory inhibitor KCN and the mitochondria uncoupler FCCP, the increase in [Ca\(^{2+}\)\(_{\text{mit}}\)] evoked by melittin was reduced by 82% (n = 11). By contrast, KCN and FCCP did not affect the melittin-induced change in [Ca\(^{2+}\)\(_{\text{mit}}\)], which remained at 1.0 ± 0.6 \(\mu M\) (n = 15).

A low dose of melittin was used to illustrate the Ca\(^{2+}\) transporting abilities of the mitochondrion in T. brucei when Ca\(^{2+}\) in the environment was lower than the proposed set point of 700 nM (4). Treatment with 125 nM melittin produced a transient injection spike followed 7 s later by a barely detectable change in [Ca\(^{2+}\)\(_{\text{mit}}\)] to a new value that was just 410 ± 100 nM (n = 13) (Fig. 4A). By contrast, [Ca\(^{2+}\)\(_{\text{mit}}\)] became transiently elevated with a broad peak of 4.8 ± 1.9 \(\mu M\), which was 11-fold higher than the level of [Ca\(^{2+}\)\(_{\text{cyt}}\)] (Fig. 4B). These data demonstrate that the mitochondrion of T. brucei is extremely sensitive to small changes in [Ca\(^{2+}\)\(_{\text{mit}}\)] when Ca\(^{2+}\) entry is induced across the plasma membrane. The total amount of free Ca\(^{2+}\) that enters the mitochondrion is approximately three times greater than the total amount of Ca\(^{2+}\) that accumulates in the cytosol.

Transfer of Ca\(^{2+}\) from an Acidic Compartment to the Mitochondrion—Experiments were initiated to determine if the mitochondrion responded with equal sensitivity when Ca\(^{2+}\) was released from an internal storage site as when Ca\(^{2+}\) entry was induced across the plasma membrane. In T. brucei, an acidic compartment contains a large pool of exchangeable Ca\(^{2+}\). Both nigericin (7) and monensin (24) were shown to release Ca\(^{2+}\) from this acidic compartment. The mechanism involves H\(^+\) influx from the compartment coupled with Ca\(^{2+}\)/H\(^+\) exchange to restore the pH gradient (24). In the present study, cells were treated with 2 \(\mu g/mL\) monensin in the presence of 5 mM EGTA to chelate the extracellular Ca\(^{2+}\). Lower resting levels of Ca\(^{2+}\) in both mitochondria and cytosol were observed with EGTA. The [Ca\(^{2+}\)\(_{\text{cyt}}\)] and [Ca\(^{2+}\)\(_{\text{mit}}\)] were 260 ± 50 nM (n = 16) and 340 ± 30 nM (n = 18), respectively. Upon addition of 2 \(\mu g/mL\) monensin, [Ca\(^{2+}\)\(_{\text{cyt}}\)] was elevated to 400 ± 50 nM (n = 9) and then returned to the basal level (Fig. 5A). By contrast, [Ca\(^{2+}\)\(_{\text{mit}}\)] was altered dramatically by monensin, rising from the resting level to 3.5 ± 1.3 \(\mu M\) (n = 16) (Fig. 5B). The peak value of [Ca\(^{2+}\)\(_{\text{mit}}\)] was approximately 7-fold higher than [Ca\(^{2+}\)\(_{\text{cyt}}\)]. These data demonstrate that the release of Ca\(^{2+}\) from the acidic compartment changes [Ca\(^{2+}\)\(_{\text{mit}}\)] much more than [Ca\(^{2+}\)\(_{\text{cyt}}\)]. The channeling of Ca\(^{2+}\) from the acidic compartment to the mitochondrion may represent a chemical signaling process in which organelles can communicate with each other by inducing selective changes in the Ca\(^{2+}\) content.

To show that the transient properties of Ca\(^{2+}\) influx into the mitochondrion were not the result of damage to the mitochondrion and to observe changes in cellular homeostasis after the
acidic compartment was compromised with monensin, melittin was added to cells after the monensin injection. If the mitochondrial membrane is damaged by monensin, it would not efficiently transport Ca\(^{2+}\) when melittin is applied. For these experiments 1 mM Ca\(^{2+}\) was added to the medium (Fig. 6). The injection of 2 µg/ml monensin caused a transient peak in [Ca\(^{2+}\)]\(_{cyt}\) to 560 ± 100 nM (n = 13) (Fig. 6A), and the second addition of 200 nM melittin elevated [Ca\(^{2+}\)]\(_{cyt}\) to the peak value of 1.7 ± 0.9 µM (n = 13). This value of [Ca\(^{2+}\)]\(_{cyt}\) is 1.4-fold higher than is observed with melittin alone (Fig. 3B). At the...
same time, monensin caused a transient rise in $[\text{Ca}^{2+}]_{\text{mit}}$, which peaked at $4.4 \pm 1.1$ µM ($n = 8$) (Fig. 6B). After the $[\text{Ca}^{2+}]_{\text{mit}}$ returned to a steady level, the second injection of 200 nM melittin increased $[\text{Ca}^{2+}]_{\text{mit}}$ to an average peak value of $7.3 \pm 1.3$ µM ($n = 9$), which indicated that the mitochondrion was not damaged by the monensin treatment.

Overall, these data demonstrate that large quantities of Ca$^{2+}$ selectively accumulate in the mitochondrion when homeostasis is disrupted by Ca$^{2+}$ influx across the plasma membrane or Ca$^{2+}$ is released from an acidic compartment. The total amount of Ca$^{2+}$ in the mitochondrion at these times exceeds the quantity in the cytoplasm. These data are consistent with a central role for the mitochondrion in maintaining Ca$^{2+}$ homeostasis in the primitive protozoan, T. brucei. In addition, Ca$^{2+}$ transfer to the mitochondrion from the acidic compartment indicates a mechanism by which one organelle might use chemical messengers to regulate the activity of another compartment.

**DISCUSSION**

Ca$^{2+}$ homeostasis is critical for cell survival. Homeostatic pathways protect the cell from toxic effects of excess Ca$^{2+}$ and serve as the source of Ca$^{2+}$ pulses during the signal process. In the present study, targeted aequorins were used to directly measure Ca$^{2+}$ concentrations inside the cellular organelles of live trypanosomes. This approach allows the dynamic interactions between homeostatic compartments to be studied. A mammalian targeting sequence was shown to efficiently target the Ca$^{2+}$-sensitive photoprotein aequorin to the mitochondrion of T. brucei. It has recently been shown that a yeast presequence and sequences from trypanosome proteins can also direct reporter proteins into the trypanosome mitochondrion (37, 43–45). Transport requires ATP, a membrane potential and a protein component on the mitochondrial surface (37, 43). Our study shows that the human localization sequence also works in trypanosomes and that the signal sequence is not proteolytically processed upon entry into the mitochondrial matrix space. Evidence of correct localization comes from: (a) immunoblots, (b) immunolocalization, (c) inhibition of aequorin luminescence with FCCP and KCN, and (d) the quantitatively different signal from CYT-AEQ and MT-AEQ, suggesting that they are not in the same compartment of the cell.

Aequorin luminescence can be converted into calculated values for free Ca$^{2+}$ concentrations using look-up tables or kinetic equations (11–15, 29–34). We chose an equation described by others (14) because it generated the lowest calculated values for $[\text{Ca}^{2+}]$. However, this equation is likely to overestimate the free Ca$^{2+}$ concentration around 200 nM, because the slope of the curve loga versus $p\text{Ca}^{2+}$ decreases in this concentration range. Consequently, the values reported with aequorin for basal levels of $[\text{Ca}^{2+}]_{\text{mit}}$ are higher than those we report using the Fura-2 system (typically in the range of 50–100 nM) (7).

The resting level of Ca$^{2+}$ within the mitochondrion varied from 340 to 400 nM depending upon whether the medium was supplemented with EGTA or Ca$^{2+}$, respectively. The large single mitochondrion has been estimated by others to occupy approximately 25% of the total cell volume in procyclic cells (42). Consequently, if all of the mitochondrial free Ca$^{2+}$ were to equilibrate with the cytosol, it would only elevate $[\text{Ca}^{2+}]_{\text{mit}}$ by around 25 nM. The low level of $[\text{Ca}^{2+}]_{\text{mit}}$ in the resting cell precludes this organelle as a source of Ca$^{2+}$ for regulatory purposes. However, we demonstrate that the trypanosome mitochondrion in vivo can effectively accumulate Ca$^{2+}$, even
when $[Ca^{2+}]_{cyt}$ is below the set point of 700–800 nm (4). Similar results have been obtained with mammalian cells by monitoring in situ changes in dihydro-Rhod 2 fluorescence (46) or using targeted aequorins (11–14). Isolated mitochondria also exhibit the ability to respond rapidly to small $Ca^{2+}$ pulses (47). The shunting of $Ca^{2+}$ to the trypanosome mitochondrion occurred whether the $Ca^{2+}$ pulse originated from the plasma membrane or from an acidic storage compartment. The mechanism of shunting probably involves the sensing of elevated microdomains in the vicinity of the plasma membrane or acidic compartment. The channeling of $Ca^{2+}$ from the ER to the mitochondrion has been observed in HeLa and other mammalian cells (12, 13). The extent of $Ca^{2+}$ shunting depended upon the proximity of the mitochondrion to the ER membrane (14). ER $Ca^{2+}$ transport has been reported in permeabilized $T. brucei$, and a SERCA cDNA encoding a thapsigargin-sensitive ATPase has been cloned (4, 6). However, thapsigargin only released small quantities of $Ca^{2+}$ from the ER in situ (5). In the present study, thapsigargin did not affect $[Ca^{2+}]_{nat}$ as occurs in mammalian cells (data not shown).

The reason why the trypanosome mitochondrion transports $Ca^{2+}$ is not altogether clear. In mammalian cells, $Ca^{2+}$-sensitive dehydrogenases are found in the mitochondrion and $Ca^{2+}$ import may be a mechanism of stimulating energy metabolism in preparation for a change in cell activity (48). This situation may apply to $T. brucei$ procyclic forms where a complete complement of mitochondrial enzymes occur. However, in bloodstream forms, mitochondrial $Ca^{2+}$ transport has still been reported (4), although no functional dehydrogenases are found. Therefore, $Ca^{2+}$ transport into the mitochondrion of these very primitive organisms may regulate other process or be exclusively for the purpose of $Ca^{2+}$ homeostasis.

Overall, the present report illustrates the dynamic interplay between homeostatic organelles within this important pathogen. During the signal process, the vast majority of $Ca^{2+}$ accumulates transiently in the mitochondrion until the $[Ca^{2+}]_{nat}$ saturates around 8 µM. Release of $Ca^{2+}$ from the mitochondrion does not elevate $[Ca^{2+}]_{cyt}$ due in part to the activity of the acidic compartment. Nonetheless, even in the presence of melittin and monensin, $[Ca^{2+}]_{cyt}$ still returns to the basal level (Fig. 5A), suggesting that other energy-dependent homeostatic organelles compensate when one system is disrupted. Nosqueftered $Ca^{2+}$ accumulates in the cytosol, and a portion of this $Ca^{2+}$ moves into the nucleus (35).

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