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Mitochondria Positioning Controls Local Calcium Influx in T Cells

Christian Schwindling,* Ariel Quintana,*1 Elmar Krause,† and Markus Hoth*

Formation of an immunological synapse (IS) between APC and T cells activates calcium entry through ORAI channels, which is indispensable for T cell activation. Successful proliferation and maturation of naive T cells is possible only if premature inactivation of ORAI channels is prevented. Although it is undisputed that calcium entry through ORAI channels is required for T cell function, it is not known if calcium influx is uniformly distributed over the plasma membrane or if preferential local calcium entry sites (for instance, at the IS) exist. In this study, we show that mitochondrial positioning determines the magnitude of local calcium entry anywhere in the plasma membrane by reducing local calcium-dependent channel inactivation: if mitochondria are close to any given local calcium entry site, calcium influx is large; if they are not close, calcium influx is small. Following formation of the IS, mitochondria are preferentially translocated to the IS in a calcium influx-dependent manner but independent of the exact calcium influx site. Mitochondrial enrichment at the IS favors local calcium entry at the IS without the necessity to enrich ORAI channels at the IS. We conclude that local calcium entry rather than global calcium entry is the preferential mechanism of calcium entry at stable ISs in Th cells. The Journal of Immunology, 2010, 184: 184–190.

The stepwise activation of Th cells is required for an effective adaptive immune response to fight against foreign Ags. This activation is initiated following the transport of foreign Ags to lymph nodes by APCs. These Ags are coupled to class II MHC molecules at the surface of APCs and corresponding naive Th cells can bind to these complexes. After this initial step, many molecular rearrangements occur in Th cells, and the highly organized immunological synapse (IS) develops between Th cells and APC (1–4).

Formation of the IS is the trigger for several signaling pathways in Th cells, which finally results in secretion of cytokines (e.g., IL-2), clonal expansion, and differentiation of the Th cells (2, 5). An indispensable step during Th cell activation is the Ca2+ entry across the plasma membrane through the opening of Ca2+ release-activated Ca2+ (CRAC)/ORAI1 channels (6–9). These channels are opened and act as Ca2+ buffers (12–19). It has previously been shown that prevent Ca2+-dependent inactivation of CRAC/ORAI1 channels, mitochondria are able to take up the inflowing Ca2+ very efficiently (10, 11). To prevent Ca2+-dependent inactivation of CRAC/ORAI1 channels, mitochondria are able to take up the inflowing Ca2+ very efficiently and act as Ca2+ buffers (12–19). It has previously been shown that mitochondria translocate toward the IS during Th cell or NK cell activation (18, 20). This mechanism results in higher intracellular Ca2+ concentrations and more effective Th cell activation and proliferation (18, 21, 22). Interestingly, mitochondria can also accumulate at the uropod of T cells during migration and facilitate lymphocyte chemotaxis (23). The role of both Ca2+ signals for the regulation of gene expression in T cells (24) and subsequent T cell activation and proliferation (21) is well established. Whether there is a physiological function for local Ca2+ entry in T cells is presently unclear. In neurons, localized Ca2+ entry at the synapse regulates exocytosis and synaptic transmission. Th cells use two different mechanisms to secrete different types of vesicles at different locations: one fraction including IL-2 and IFN-γ is secreted at the IS, whereas the other one including TNF and the chemokine CCL3 is secreted everywhere at the plasma membrane (25). Huse et al. (25) have shown that different transport proteins are associated with each pathway and have therefore proposed that different molecular mechanisms are responsible for the two modes of secretion. Like for most types of exocytosis, it can be safely assumed that Ca2+ signals modulate the rates of vesicle secretion. Local Ca2+ influx at the IS may therefore regulate the IS-dependent secretory pathway, whereas the global secretion pathway may depend on global Ca2+ signals.

Data about local Ca2+ influx in T cells are sparse. Lioudyno et al. (26) reported local Ca2+ entry at the IS due to ORAI1 and STIM1 enrichment at the IS (26), whereas Barr et al. (27) reported ORAI1 and STIM1 enrichment in different areas, including the IS but also in distal capping areas. In the current study, we have analyzed the influence of mitochondrial positioning and local Ca2+ entry on each other in Th cells.

Materials and Methods

Cells

Human Jurkat T cell lines were grown in RPMI 1640 medium supplemented with 10% FCS and penicillin-streptomycin as described previously (28). Cells were continuously maintained in log-phase growth at 37°C with 5% CO2. For the experiments, the diphtheria toxin-resistant version of the parental cell line generated by Fanger et al. (29) was used. CD4+ T cells were prepared and maintained as previously described (21). All experiments have been approved by the local ethics committee.
Reagents

The reagents used in our experiments include: thapsigargin (TG; stock 1 mM in DMSO, Invitrogen, Karlsruhe, Germany) anti-human CD3 (obtained from Abd Serotec, Düsseldorf, Germany), and anti-human CD8 mAbs (BD Bioscience, Heidelberg, Germany). All chemicals not specifically mentioned were from Sigma-Aldrich, München, Germany (highest grade).

Bead stimulation and colabeling with fura 2-AM and MitoTracker Green FM

All procedures were carried out exactly as described previously (18).

Fluorescence microscopy, Ca^{2+} imaging, and rhod-2 measurements

Ca^{2+} and mitochondrial imaging were carried out as described (18) with a 60× (UPlanFL, Olympus, Melville, NY; numerical aperture = 1.25, oil) objective. The excitation wavelengths for fura 2 were 340 and 380 nm; MitoTracker (Invitrogen) was excited at 490 nm. TG (1 μM) or anti-human CD3/CD28 mAb coated beads were used to stimulate Jurkat T cells. m-carbonyl cyanidem-chlorophenylhydrazone (CCCP) (1 μM) was used for disrupting mitochondrial Ca^{2+} uptake. Rhod-2 measurements were carried out exactly as described previously (18).

Combined fluorescence microscopy and patch clamp

Cells were loaded with 2 μM fura 2-AM and 200 nM MitoTracker Green FM simultaneously for 30 min at room temperature. After two washing steps, cells were kept in Ca^{2+}-free solution containing 1 μM TG to deplete Ca^{2+} stores irreversibly and activate CRAC channels. To induce local Ca^{2+} influx in T cells, a cell-attached patch was then formed with the patch pipette containing 1 mM Ca^{2+} Ringer solution. The membrane potential was changed from +150 mV to −100 mV for Jurkat T cells and +60 mV to −60 mV for CD4+ T cells to induce Ca^{2+} influx via the patch pipette. Fura 2 ratios and mitochondrial localization were detected simultaneously using an Olympus IX70 microscope with a 60× UPlanApo objective (Olympus; numerical aperture = 1.25, oil immersion). A TILL monochromator (TILL Photonics, Gräfelfing, Germany) was used for excitation; the dichroic mirror was a 500 LP and the emission filter a 515 LP.

Actin staining

T cells were washed two times with PBS (pH 7.4), fixed in 3.7% formaldehyde solution for 10 min at room temperature, washed two or more times, and stained with Texas Red-X phalloidin for 30 min. Actin microfilaments were imaged on an inverted microscope with a 10× objective (Nikon, Tokyo, Japan).
times with PBS, treated with 0.1% Triton X-100 for 3–5 min, washed two or more times with PBS, and stained with Texas Red-X phallolidin (catalog no. T7471; Invitrogen) for 20 min at room temperature. After the staining period, cells were washed at least twice before taking pictures. The staining procedure was performed on the stage of the microscope after the combined patch clamp/imaging experiments were finished.

Data analysis

Data were analyzed using TILL Vision (TILL Photonics), Igor Pro (WaveMetrics, Lake Oswego, OR) and Microsoft Excel (Microsoft, Redmond, WA). All values are given as mean ± SEM (n, number of cells). If data points were normally distributed, a paired or unpaired two-sided Student t test was used. If normal distribution could not be confirmed, a nonparametrized test (Mann-Whitney) was carried out.

Results

Ca\(^{2+}\) influx is necessary for the translocation of mitochondria to the IS

We have previously shown that Ca\(^{2+}\) influx through CRAC channels regulates the translocation of mitochondria to the plasma membrane (17) and to the IS (18) during and after its formation with Ab-coated beads. In this study, we confirm these findings and show in addition that Ca\(^{2+}\) influx is necessary for the translocation of mitochondria during the early and late phase of IS formation. Fig. 1A shows an example of anti-CD3/anti-CD28 Ab-coated bead–induced mitochondrial translocation to the IS in the presence of Ca\(^{2+}\) entry from the extracellular space. In the absence of external Ca\(^{2+}\), the release of Ca\(^{2+}\) from stores was not sufficient to promote translocation of mitochondria to the IS (Fig. 1B). The statistical analysis in the presence and absence of extracellular Ca\(^{2+}\) confirms that Ca\(^{2+}\) influx is needed for mitochondrial translocation to the IS (Fig. 1C, 1D). However, Ca\(^{2+}\) influx is necessary but not sufficient because in the absence of actin cytoskeleton rearrangement during IS formation, Ca\(^{2+}\) influx alone was not able to induce mitochondrial transport to the IS (18). The same is true for the rearrangement of the actin cytoskeleton, because actin fibers moved to the IS in the absence of extracellular Ca\(^{2+}\) (Fig. 1E), and mitochondrial translocation toward the IS was completely absent (Fig. 1B, 1D). In conclusion, both the actin rearrangement and Ca\(^{2+}\) entry are necessary for mitochondrial accumulation at the IS; none of the two can therefore be sufficient.

Local Ca\(^{2+}\) entry in T cells

To test whether local Ca\(^{2+}\) entry at the IS was required to translocate mitochondria to the IS, we applied a technology previously used in pancreatic acinar cells to induce local Ca\(^{2+}\) entry (30). A cell-attached patch was formed on a cell bathed in 0 mM Ca\(^{2+}\) solution, which contained 1 μM TG to deplete intracellular Ca\(^{2+}\) stores and activate store-operated CRAC channels. The patch pipette contained 1 mM Ca\(^{2+}\) and was thus the only extracellular Ca\(^{2+}\) source (Fig. 2A). The patch was clamped at +150 mV immediately after establishing the on-cell configuration to avoid any Ca\(^{2+}\) influx over the patch. A patch potential change from +150 mV to −100 mV induced Ca\(^{2+}\) entry over the patch, resulting in a global Ca\(^{2+}\) signal in the T cell (Fig. 2B). The intracellular Ca\(^{2+}\) concentration increase showed a Ca\(^{2+}\) overshoot typical for T cells and was terminated completely by depolarizing the patch membrane back to +150 mV. Ca\(^{2+}\) imaging of cells under these conditions revealed that the global Ca\(^{2+}\) signal cannot be explained by simple Ca\(^{2+}\) diffusion away from the Ca\(^{2+}\) entry source. Complicated diffusion patterns and Ca\(^{2+}\) gradients imply that diffusion barriers and/or Ca\(^{2+}\) transport through organelles must exist (Fig. 2C).

The technology of focal Ca\(^{2+}\) entry in T cells was now used to test whether Ca\(^{2+}\) influx at the IS was required to induce translocation of mitochondria to the IS. Fig. 3A shows a model T cell that is patched on the upper side of the cell. In addition, an anti-CD3/anti-CD28 Ab-coated bead is brought into contact with the cell at the opposite side to stimulate the cell and induce IS formation. IS formation was confirmed by actin cytoskeleton reorganization toward the cell-bead contact point (Fig. 3B). Using this setup, we could now separate the location of the IS formation and Ca\(^{2+}\) entry from each other. Analyzing mitochondrial movement under these conditions, we found that mitochondria were clearly moved toward the IS and not toward the Ca\(^{2+}\) entry site (Fig. 3C). In the example shown, small Ca\(^{2+}\) entry was activated by the potential change after 20 s. Translocation of mitochondria to the IS was observed immediately after activation of Ca\(^{2+}\) entry.
by the membrane potential jump during IS formation (data not shown). The qualitative analysis revealed that translocation of mitochondria to the IS was observed in 90% of the cells. Mitochondrial fluorescence was on average increased by a factor of two, whereas it was constant at the Ca$^{2+}$ entry site (Fig. 3D). We conclude that mitochondria are moved to the IS independent of the position of the Ca$^{2+}$ entry source.

**Mitochondria control local Ca$^{2+}$ entry in T cells**

We have previously shown that global Ca$^{2+}$ entry (over the whole plasma membrane) in T cells is controlled by mitochondria Ca$^{2+}$ uptake (13, 18). To test whether the same is true for local Ca$^{2+}$ entry, a cell-attached patch was formed on a cell bathed in 0 mM Ca$^{2+}$ solution, which contained 1 μM TG to deplete intracellular Ca$^{2+}$ stores and activate store-operated CRAC channels. Changing the patch potential from +150 mV to −100 mV, we found that intracellular Ca$^{2+}$ concentration increased much more in the case in which mitochondria were by chance close to the patch and the local Ca$^{2+}$ influx source compared with the case in which mitochondria were by chance far away from the patch (Fig. 4A). In the case in which mitochondria were close to the Ca$^{2+}$ influx source, the Ca$^{2+}$ influx during steady state was much bigger compared with the case in
which mitochondria were far away from the Ca^{2+} entry source (Fig. 4B). The statistical analysis reveals a clear difference between the Ca^{2+} signals in cells with mitochondria close to or away from the patch (Fig. 4C). A similar result was found in primary human T cells (Fig. 4D). The effect of mitochondria on CRAC/ORAI channel activity can be explained by capability of mitochondria to take up large amounts of Ca^{2+} (31), which enable them to reduce the local accumulation of Ca^{2+} near the sites that govern Ca^{2+}-dependent channel inactivation efficiently (13, 18). Indeed, the larger Ca^{2+} signals were reduced if mitochondrial Ca^{2+} uptake was inhibited by 1 μM CCCP in cells with mitochondria close to the patch (Fig. 4C). These experiments clearly indicate that mitochondrial positioning controls local Ca^{2+} entry in T cells.

To analyze how much Ca^{2+} was taken up by mitochondria depending on their position, we used rhod-2 loading by an electroporation technique established previously (18, 19).

FIGURE 4. Local Ca^{2+} entry is influenced by the localization of mitochondria relative to the Ca^{2+} entry site. A. Examples of two Jurkat T cells treated with TG and loaded with MitoTracker Green AM and fura-2-AM. Ca^{2+} influx was activated by changing the patch potential across the patch from +150 mV to −100 mV. The upper panel shows a Jurkat T cell where the mitochondria were by chance localized close to the patch (as seen by the MitoTracker picture excited at 470 nm); the lower panel shows a Jurkat T cell where the mitochondria were by chance localized far away from the patch (as seen by the MitoTracker picture excited at 470 nm). B. Fura 2 ratios of two Jurkat T cells under experimental conditions as in A. The patch potential was changed after 20 s from +150 mV to −100 mV. C. Average fura 2 ratios during the Ca^{2+} plateau (450–500 s) of cells with mitochondria localized close to the patch pipette (n = 19) or cells with mitochondria localized away from the patch pipette (n = 12). Values were significantly different (two-sided unpaired t test, p < 0.05). The third bar shows cells with mitochondria localized close to the patch pipette (n = 15) but in addition treated with 1 μM CCCP to disrupt the mitochondrial Ca^{2+} uptake. D. Average fura 2 ratios from 450 to 500 s of primary CD4+ T cells using the same experimental conditions as in A and B, only the patch potential was changed after 20 s from +60 mV to −60 mV (the patches of the primary cells were not stable enough at more extreme potentials). Ten cells with mitochondria close to the patch and three cells with mitochondria away from the patch were analyzed. E. Infrared and fluorescence images of a Jurkat T cell treated with TG and patched in a cell-attached patch configuration. Mitochondrial Ca^{2+} concentrations were visualized with rhod-2. Ca^{2+} influx was activated by changing the patch potential across the patch after 20 s from +150 mV to −100 mV. The arrows highlight mitochondrial clusters located near or away from the Ca^{2+} influx site. F. Statistical analysis of cells as the one shown in E. Mitochondria located near (red trace; n = 9 cells) or away (black trace; n = 7 cells) from the Ca^{2+} influx site were analyzed.
mitochondria close to the Ca$^{2+}$ influx source took up more Ca$^{2+}$ earlier and at a higher rate than the ones further away from the influx source after Ca$^{2+}$ influx was activated by the patch potential change from +150 mV to −100 mV, as evident from the pictures in Fig. 4E and the time course of the relative fluorescence signals in Fig. 4F.

Finally, we induced IS formation and local Ca$^{2+}$ influx at the same location. To do this, we included anti-CD3/anti-CD28 Abs in the patch pipette (Fig. 5A). Again, a cell-attached patch was formed with Ca$^{2+}$ included in the patch solution but no Ca$^{2+}$ in the bath solution. IS formation at the position of the cell-attached patch under these conditions was confirmed by actin cytoskeleton rearrangement (Fig. 5B). Comparing the many cells that had mitochondria localized close to the IS with the few ones that had mitochondria localized away from the IS and the focal Ca$^{2+}$ entry, we found again that Ca$^{2+}$ signals were significantly higher if mitochondria were close to the IS (Fig. 5C, 5D). In summary, we conclude that mitochondrial positioning determines where focal Ca$^{2+}$ entry is induced. Following their accumulation at the IS, mitochondria therefore induce local Ca$^{2+}$ entry through CRAC/ORAI channels at the IS.

Discussion

We have shown that Ca$^{2+}$ influx anywhere in the plasma membrane is required to induce the accumulation of mitochondria at the IS. Mitochondrial positioning allows local Ca$^{2+}$ entry only at sites where mitochondria are localized. Therefore, we have shown that sustained Ca$^{2+}$ entry in T cells, which is essential to induce and maintain NFAT translocation in the nucleus, occurs preferentially at the IS.

In mast cells, local Ca$^{2+}$ entry is coupled to specific cellular functions like 5(S)-hydroxy-6(R)-S-glutathionyl-7,9-trans-11,14-cis-eicosatetraenoic acid secretion (32) or gene expression (33, 34). This further highlights the potential importance of localized Ca$^{2+}$ entry in immune cells to control secretion and gene expression. One potential mechanism to generate local Ca$^{2+}$ entry at a given site of a cell is the enrichment of STIM and ORAI at a given location. Lioudyno et al. (26) found that STIM1 and ORAI1 were accumulated at the IS in T cells following their formation. This accumulation favored Ca$^{2+}$ entry at the IS and higher Ca$^{2+}$ signals in the subcellular areas close to the IS. However, using high-resolution microscopy, Barr et al. (27) reported a diverse localization of STIM1 and ORAI1 throughout the plasma membrane after IS formation. They found that STIM1 and ORAI1 often clustered in caps, which were often localized at the distal pole and not at the IS. In addition, they also found that caplike structures were able to move to sites close to the IS. This dynamic led Barr et al. (27) to speculate that the caps could be used to assemble pre-existing ORAI1–STIM1 complexes for use at newly established synapses. Alternatively, the caps could also prevent STIM1–ORAI1 complexes to participate actively in Ca$^{2+}$ entry outside the IS. Unfortunately, it cannot be finally resolved at the moment if and where ORAI and STIM are localized during and after IS formation. Because we have shown that mitochondrial positioning determines local Ca$^{2+}$ entry at the IS, only ORAI1 channels at the IS would stay open for extended times. Thus, the mitochondrial localization at the IS induces localized and sustained Ca$^{2+}$ influx.

In many cell types, it has been demonstrated that mitochondria can sense microdomains of Ca$^{2+}$ released from the endoplasmic reticulum (ER) (31). The close interaction between ER and mitochondria influences Ca$^{2+}$ release from the ER and Ca$^{2+}$-dependent cell functions. In T cells, mitochondria appear not to have such a drastic role in controlling Ca$^{2+}$ release from the ER (12, 28). Furthermore, we could not detect an influence of mitochondrial mobility on Ca$^{2+}$ release from the ER (18). In conclusion, Ca$^{2+}$ release from the ER appears not to have the same functional importance in T cells as in other cells (16), highlighting the role of Ca$^{2+}$ entry at the IS as the major source of Ca$^{2+}$ (22).

The duration of an IS can range from seconds or minutes up to several hours (35, 36). Naive CD4$^+$ or CD8$^+$ cells have to continuously scan their environment to find matching APCs. Very short IS durations are beneficial to allow as many contacts as possible to find a potentially rare match with an Ag-presenting dendritic cell. Because T cells still show net migration during the formation of such a short IS, Dustin (37) termed such a short-lived synapse “kinapse” (from “kinetic synapse”). Kinapses have been implicated to be important in the induction of tolerance in case naive T cells only contact low-potency MHC-bound Ags. T cells did not change migration pattern under these conditions and were never arrested to form a stable long-lasting IS, but they finally became tolerant after forming several kinapses (36). Longer IS durations of 20–60 min have been observed when CTLs kill target cells (38). Even longer lasting stable synapses are required for the maturation and proliferation of naive T cells.

**FIGURE 5.** Local Ca$^{2+}$ entry at the IS is influenced by the localization of mitochondria with respect to the Ca$^{2+}$ entry site at the IS. A, Experimental setup: same as in Fig. 2A, only that in addition, an IS is induced at the site of the patch by including anti-CD3/anti-CD28 Abs in the patch pipette. B, Infrared and fluorescence images from a Jurkat T cell. The actin cytoskeleton was stained by Texas Red-X phalloidin after 30 min of stimulation with the patch pipette. The membrane potential across the patch was changed after 20 s from +150 mV to −100 mV. C, Fura 2 ratios of two Jurkat T cells under experimental conditions as in A with mitochondria localized close or away from the patch. The latter case was extremely rare because in >90% of the cells, mitochondria were translocated to the IS (compare to Fig. 3C). D, Average fura 2 ratios during the Ca$^{2+}$ plateau (450–500 s) of cells as in C with mitochondria close to the patch (n = 15) or with mitochondria away from the patch (n = 3).
importance of Ca\textsuperscript{2+} signals for all aspects of T cell activation, mitochondrial positioning may be very important to determine synaptic efficacy by regulating ORAI1 activity.

Mitochondria-dependent restricted Ca\textsuperscript{2+} influx at the IS would preferentially induce Ca\textsuperscript{2+} signals close to the IS. This may initially restrict Ca\textsuperscript{2+}-dependent vesicle exocytosis to the area of the IS. However, if the IS persists for extended times, mitochondria would also transport the Ca\textsuperscript{2+} away from the IS to distant cytosolic sites and induce vesicle release there. In the case of effector CD4\textsuperscript{+} T cells, it has been indeed shown that different mechanisms exist to release vesicles restricted either to the IS only or anywhere at the plasma membrane (25). Therefore, we believe that mitochondria-dependent local Ca\textsuperscript{2+} entry could be used to determine the place and timing of vesicle release.

In the case of naive CD4\textsuperscript{+} cells, they have to first continuously scan their environment to find matching APCs. Very short IS durations are beneficial to allow them as many contacts as possible to find a potentially rare match with an APC. However, once the right APC is found, longer IS (16–24 h) are required to sustain local Ca\textsuperscript{2+} influx through Ca\textsuperscript{2+} release-activated Ca\textsuperscript{2+} channels. In the case of naive CD4\textsuperscript{+} T cells, it has been indeed shown that different mechanisms exist to release vesicles restricted either to the IS only or anywhere at the plasma membrane (25). Therefore, we believe that mitochondria-dependent local Ca\textsuperscript{2+} entry could be used to determine the place and timing of vesicle release.

References

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Disclosures

The authors have no financial conflicts of interest.