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Mitochondrial regulation of synaptic plasticity in the hippocampus

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SUMMARY

Synaptic mechanisms of plasticity are calcium dependent processes that are affected by dysfunction of mitochondrial calcium buffering. Recently, we observed that mice deficient in mitochondrial voltage-dependent anion channels, the outer component of the mitochondrial permeability transition pore, have impairments in learning and hippocampal synaptic plasticity, suggesting that the mitochondrial permeability transition pore is involved in hippocampal synaptic plasticity. In this study, we examined the effect on synaptic transmission and plasticity of blocking the permeability transition pore with low doses of cyclosporin A and found a deficit in synaptic plasticity and an increase in baseline synaptic transmission. Calcium imaging of pre-synaptic terminals revealed a transient increase in the resting calcium concentration immediately upon incubation with cyclosporin A that correlated with the changes in synaptic transmission and plasticity. The effect of cyclosporin A on pre-synaptic calcium was abolished when mitochondria were depolarized prior to cyclosporin A exposure, and the effects of cyclosporin A and mitochondrial depolarization on pre-synaptic resting calcium were similar, suggesting a mitochondrial locus of action of cyclosporin A. To further characterize the calcium dynamics of the
mitochondrial permeability transition pore, we used an *in vitro* assay of calcium handling by isolated brain mitochondria. Cyclosporin A-exposed mitochondria buffered calcium more rapidly and subsequently triggered a more rapid mitochondrial depolarization. Similarly, mitochondria lacking the voltage dependent anion channel 1 isoform depolarized more readily than littermate controls. The data suggest a role for the mitochondrial permeability transition pore and voltage-dependent anion channels in mitochondrial synaptic calcium buffering and in hippocampal synaptic plasticity.

**INTRODUCTION**

The mitochondrial permeability transition pore (MPT) is a complex believed to be composed of the voltage-dependent anion channel (VDAC) in the mitochondrial outer membrane, the adenine nucleotide transporter in the inner membrane and cyclophilin-D (Cyp-D) in the matrix, and is found in mitochondria of all eukaryotic cells. Although the majority of research on the function of the MPT and its components has focused on apoptosis (1-3), the MPT has recently been shown to play a role in learning and synaptic plasticity in mice (4) as well as in other physiological cellular functions (5). In both pathological and physiological functions, the induction of the MPT is mediated by mitochondrial calcium influx above a certain threshold, and, once opened, the MPT conducts small substrates and ions out of the mitochondrial matrix (6). It has been therefore proposed that
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following physiologic calcium influx into the mitochondrial matrix, formation of the MPT might be a physiological mechanism of mitochondrial calcium release (5).

We are interested in determining the role of mitochondrial calcium regulation in synaptic function. The most extensively characterized synapses in the mammalian CNS are the Schaffer collateral synapses between CA3 pyramidal axons and their post-synaptic targets on the dendrites of CA1 pyramidal neurons. As mitochondria are typically found in CA3 presynaptic terminals as well as CA1 dendrites, we hypothesized that CA3-CA1 hippocampal long-term potentiation (LTP) and paired-pulse facilitation (PPF) would be sensitive to alterations in mitochondrial calcium regulation by the MPT since both depend on calcium-dependent processes within synaptic terminals.

In this study, we examined the effect of blocking the MPT on LTP and PPF using low doses of cyclosporin A (CsA). In addition, we monitored changes in calcium dynamics caused by CsA by imaging pre-synaptic terminals loaded with the fluorescent calcium indicator FURA-2. We found that blocking the MPT caused a transient increase in resting calcium in pre-synaptic terminals, correlating with changes in synaptic transmission and plasticity. The increase caused by CsA was abolished by depolarizing mitochondria with tetraphenylphosphate (TPP+) prior to CsA exposure indicating a common target, i.e., mitochondria. In addition, the characteristics of the effects of TPP+ and CsA on resting calcium were similar suggesting the possibility of a common mechanism of action.
To more precisely determine the effect of blocking the MPT on mitochondrial calcium handling we employed an *in vitro* assay to study calcium handling in isolated brain mitochondria and found that CsA induces earlier and more rapid mitochondrial depolarization and subsequent calcium release, which may explain the transient increase of calcium in presynaptic terminals. Similarly, VDAC1-deficient mitochondria, depolarized and subsequently released their mitochondrial calcium stores more readily than wild-type littermate controls, which may explain their similar electrophysiological deficits (4). Based on these results we propose a model to explain how the MPT serves to help prevent mitochondrial calcium overload and subsequent depolarization. Overall, our studies are consistent with an important role for synaptic mitochondria in calcium regulation, and specifically suggest a role for the MPT in neuronal calcium regulation.

**METHODS**

**Hippocampal Slice Physiology.** Hippocampal slices (400 µm) were prepared as previously described (7). Hippocampal slices were bathed (1ml/min) with artificial cerebrospinal fluid (125 mM NaCl, 2.5 mM KCl, 1.24 mM NaH$_2$PO$_4$, 25 mM NaHCO$_3$, 10 mM D-glucose, 2 mM CaCl$_2$, 1 mM MgCl$_2$ and 10 µM cyclosporin A as indicated) in an interface chamber maintained at 30°C bubbled with 95% O$_2$/5% CO$_2$. The Schaffer collateral axons were stimulated with a
bipolar electrode and the field excitatory post-synaptic potential (fEPSP) was recorded in the stratum radiatum (SR) of area CA1. Responses were monitored every 20-30 seconds for 20 minutes to ensure a stable baseline. Measurements are shown as the average slope of the fEPSP from 4-6 individual traces and are normalized to 10 minutes of baseline recordings. Baseline stimulus intensities were adjusted to produce an fEPSP at 50% of the maximal response. 100 Hz LTP was induced by stimulating twice at high frequency (100 Hz) at baseline intensity for 1 second each, 20 seconds apart. Theta burst stimulation (TBS) consisted of three trains of stimuli delivered at 20-second intervals, each train composed of 10 stimulus bursts delivered at 5 Hz, with each burst consisting of four pulses at 100 Hz. Paired-pulse facilitation (PPF) was induced by two single stimuli at baseline intensity, 30 milliseconds apart. CsA (Alexis Pharmaceuticals) was dissolved in 99% ethanol (0.1% final ethanol concentration) and added to ACSF while stirring over 10 minutes to increase solubilization. Thorough rinsing of all plastic tubes and parts exposed to CsA with ethanol and water prevented contamination of control slices.

**Hippocampal Calcium Imaging.** Hippocampal slices were prepared as described above, submerged in artificial cerebral spinal fluid and maintained with 95% O$_2$/5% CO$_2$ bubbling at 30°C. As described in detail in Wu and Saggau (8), Fura-2 AM (Molecular Probes) dissolved in 80:20 DMSO:pluronic acid was pressure-injected in the SR of area CA1 where the AM-ester dye is taken up by CA3 axons, the AM-ester cleaved, and the cell-impermeable Fura-2 transported
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to the presynaptic terminals. An area in SR of CA1 about 1mm away from the
injection site was illuminated at a single excitation wavelength, 340 nm or 380 nm
as indicated. Fluorescence was collected with a 50X objective, filtered by a long-
pass filter (490 nm) and converted into an electrical signal by a single
photodiode. Resting presynaptic calcium was determined by ratiometric
measurements of fluorescence excited at both 340 nm and 380 nm in the
absence of CA3 stimulation. For PPF measurements, two traces recorded 1
minute apart were averaged to improve the signal-to-noise ratio.

**Western Blotting.** Hippocampal slices were prepared and incubated in an
interface chamber as described above. High doses of CsA (250 µM), low doses
of CsA (10 µM) or no CsA in 0.5% Tween-20/0.5% ethanol was added to the
ACSF for 30 minutes. Slices were removed from the interface chamber and
homogenized in buffer containing 20 mM Tris, 1 mM EGTA, 1 mM EDTA, 1 mM
Na₄P₂O₇, protease inhibitor cocktail (Sigma), 1 mM p-nitrophenyl phosphate,
0.25 mM Na₃VO₄, pH 7.5, at 4ºC. Homogenates were run on a 12% SDS-PAGE,
transferred to a nylon membrane (Millipore) and probed with phosphospecific
antibodies to phosphorylated synapsin I (a-Ser603 and a-Ser62/67
phosphospecific antibodies were generous gifts from the lab of Paul Greengard;
a-Ser553 was purchased from Santa Cruz Biotechnology) and total synapsin (a-
synapsin was purchased from Cell Signaling Technology). Blots were developed
using the ECL system (Amersham Pharmaceuticals). Each experiment was
repeated at least three times.
Hippocampal ATP concentration. Mouse hippocampal slices were prepared and maintained as previously described for electrophysiological experiments. Slices were exposed to 50 mM KCl for 10 minutes, 2 minutes or none before perchlorate extraction. A luciferase based assay from Molecular Probes was used to quantify the ATP concentration and normalized to protein concentration, as determined by BCA protein assay (Pierce).

Mitochondrial Isolation from Mouse Brain. Mouse brain mitochondria were isolated by Percoll centrifugation outlined in (9). One mouse forebrain was minced on ice and homogenized in a small glass homogenizer with 3 ml of a 12% Percoll solution in isolation buffer composed of 0.32 M sucrose, 1 mM K-EGTA, 10 mM Tris, pH 7.1. The Percoll homogenate was carefully added on top of 3.5 ml of 26% Percoll, which was layered over 3.5 mL of 40% Percoll in a tube and centrifuged at 30,700 x g for 5 minutes at 4 degrees. The cloudy layer at the interface of the lower two Percoll solutions was aspirated, washed 1:4 (v/v) with isolation buffer, and centrifuged at 16,700 x g for 10 minutes at 4 degrees. The pellet was resuspended in 1% BSA (in isolation buffer) and spun at 8,000 x g for 12 minutes at 4 degrees. The pellet was then washed in 900 µl of BSA-free isolation buffer, centrifuged at 8,000 x g for 12 minutes at 4 degrees and finally resuspended in 750 µl of BSA-free isolation buffer. Isolated mitochondria were tested for respiration using an oxygraph (biological oxygen monitor, YSI Model 5300) and a Clark-type electrode (oxygen probe, YSI Model 5331) as described...
mitochondria with a respiratory ratio of >2 on succinate were used for the \textit{in vitro} assays. In addition, only mitochondria which possessed an intact outer membrane, as tested indirectly by the cytchrome C assay (11), were used. Isolated mitochondria stored on ice can be reliably used for experiments for up to 3 hours.

\textbf{In Vitro Assay for Mitochondrial Calcium Handling.} 300 µl of buffer (125 mM mannitol, 75 mM sucrose, 10 mM KCl, 10 mM Tris-HCl, 5 mM KH$_2$PO$_4$, 5 mM glutamic acid, 5 mM pyruvic acid, 5 mM succinic acid, 5 mM malic acid, pH adjusted with KOH to 7.3, osmolarity = 300 mOsm) was pre-warmed in a stirring measurement chamber for 1 minute at 32 °C. To the warming buffer, ADP was added to a final concentration of 100 µM and CsA (dissolved in 99\% EtOH), or an equivalent volume of EtOH for control, was added to a final concentration of 1 µM as indicated. Mitochondria were added to a final protein concentration of 60-70 µg/ml (approximately 50 µL of mitochondrial preparation) and incubated for 2 minutes to warm up to 32 °C. Extramitochondrial calcium was monitored using a calcium-selective electrode (World Precision Instruments) and recorded every 20 seconds. A CaCl$_2$ bolus was added to reach the desired calcium concentration as indicated. CsA, and other drugs, were either added 2 minutes before the calcium bolus or after calcium was maximally buffered, defined as 40 seconds of zero net flux. Ruthenium Red (RuR), an inhibitor of the mitochondrial calcium uniporter, carbonyl cyanide 3-chlorophenylhydrazone (CCCP), a mitochondrial proton uncoupler, and tetraphenylphosphate (TPP$^+$) were purchased from Sigma.
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In these experiments, we observed that there is a range of calcium release rates that varies between mitochondrial preparations and depends partly on the initial calcium bolus, mitochondrial concentration, the background strain of mouse and the degree of mitochondrial depolarization. The traces we show represent the average release rate for one set of experiments, where each set was run at least three times with different mitochondrial preparations.

RESULTS

Approximately half of all CA3 pre-synaptic terminals contain at least one mitochondrion (Ventura and Harris, 1999) and mitochondria are also typically found in dendrites of CA1 pyramidal neurons (Figure 1). We investigated the role of these mitochondria in synaptic transmission and plasticity.

**CsA induced changes in synaptic transmission and plasticity.** As previously reported in Weeber, et. al. (4), we found that 10 µM CsA significantly attenuates hippocampal 100 Hz LTP at Schaffer collateral synapses in area CA1 of the mouse hippocampus (Figure 2A). We sought to investigate the basis of this effect by assessing acute changes caused by CsA. Immediately upon addition of CsA, synaptic transmission began to increase and reach a plateau at approximately 25% above baseline (Figure 2B, also evident in the baseline of figures 2A, 2C). Interestingly, although CsA attenuated LTP induced by a 100 Hz stimulating protocol (Figure 2A), it did not appear to attenuate LTP induced by
theta burst stimulation (TBS) (Figure 2C), even when the increase in baseline synaptic transmission is subtracted from the theta-burst induced potentiation (139% potentiation in controls vs. 128% in CsA-exposed slices, p=0.11). 100 Hz stimulation (Panel A) and TBS (Panel C) are believed to differ in their mechanism of LTP induction because of differences in the characteristics of calcium influx into synaptic terminals (12), which may affect different biochemical pathways. For example, theta-burst LTP involves the MAPK pathway in the mouse hippocampus while 100 Hz LTP does not (Selcher et al., 2003). Thus our studies suggest that the effect of CsA on synaptic plasticity affects biochemical pathways that are important for 100 Hz LTP but not theta-burst LTP; however, it is important to note that the CsA-elicited increase in baseline synaptic transmission complicates interpretation of our TBS-induced LTP result.

One question that arises from our CsA experiments is whether the locus of action is mitochondrial as opposed to other potential sites of action. TPP+ has been shown to attenuate post-tetanic potentiation at the crayfish neuromuscular junction by causing pre-synaptic mitochondrial depolarization (Tang and Zucker, 1997). In the mouse hippocampal area CA1, exposure to TPP+ 20 minutes prior to a 100 Hz LTP simulation paradigm caused a marked reduction in LTP induction, not unlike CsA exposure (Figure 2D). Although the mechanisms of action of TPP+ and CsA are different, the similarity of the effect of TPP+ and CsA on early LTP insinuates a common final effect on mitochondrial calcium handling. Unlike CsA however, sustained mitochondrial depolarization by TPP+ eventually
causes swelling and alterations in metabolic activity (Figure 5) resulting in loss of synaptic activity (Figure 2D). Overall, however, our data indicating that TPP+ application blocks LTP induction in a fashion reminiscent of CsA is consistent with our interpretation that CsA acts at a mitochondrial locus.

We also assessed the effects of MPT inhibition on shorter-term forms of synaptic plasticity, in particular paired pulse facilitation (PPF). We particularly followed PPF because it is generally held to involve pre-synaptic calcium handling. The effect of CsA exposure on PPF, becoming evident approximately 20 minutes after adding CsA, was a reduction in the 2\textsuperscript{nd} fEPSP compared to the 1\textsuperscript{st} fEPSP such that PPF was attenuated (Figure 3). These findings are consistent with our previous observations of the effect of CsA on hippocampal synaptic plasticity (4).

In summary, our electrophysiologic studies indicate that CsA has both an immediate effect to increase synaptic transmission, followed by a delayed attenuation of two forms of synaptic plasticity, PPF and 100 Hz LTP. We hypothesize that the effect of CsA in synaptic terminals is in part due to inhibition of the MPT and subsequent alterations in mitochondrial calcium handling.

**Low doses of CsA did not inhibit calcineurin.** One specific concern with the use of CsA to block the MPT is the possibility of inhibition of the protein phosphatase, calcineurin. Typically, the concentrations of CsA used to block calcineurin are at least 10-25-fold greater than used in this study (13,14). In
addition, several considerations suggest that calcineurin is not inhibited in our studies. Hippocampal slices from mice lacking calcineurin exhibit enhanced PPF (15), and FK506, another calcineurin inhibitor, does not affect PPF (16). FK506 also reduces baseline synaptic transmission (16). None of these effects of calcineurin inhibition are observable in our experiments with low doses of CsA.

Nevertheless, in order to confirm that low doses (10 µM) of CsA do not inhibit calcineurin in the hippocampal slice to the same extent as high doses (250 µM) of CsA do, we used phosphospecific antibodies to detect the phosphorylation state of a calcineurin substrate, synapsin I. Hippocampal slices incubated under the same conditions as for electrophysiology, in the presence or absence of high or low doses of CsA, were harvested for Western blotting with phosphospecific antibodies to synapsin I phosphorylated at Ser603, Ser553 and Ser62/67, all of which have been shown to be sensitive to calcineurin (17). Incubation in 250 µM CsA resulted in dramatically enhanced phosphorylation of all three synapsin I phosphorylation sites which is likely due to inhibition of dephosphorylation by calcineurin (Figure 4). Equally dramatic is the lack of phosphorylation in slices exposed to 10 µM or no CsA (Figure 4). Taken together with our electrophysiological observations, these findings indicate that the low doses of CsA used in this study do not cause calcineurin inhibition and suggests that low doses of CsA are acting via a different mechanism. Among the known targets of CsA, mitochondrial cyclophilin-D has one of the lowest $K_i$ values in vitro (18) and cyclophilin-D has been shown to mediate the functional inhibition of the MPT by
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CsA in isolated mitochondria (19). In addition, our prior studies of hippocampal slices from VDAC-deficient mice showed a similar electrophysiological phenotype to CsA-exposed wild-type slices. Thus it is likely that the effect of CsA on hippocampal electrophysiology results from inhibition of the MPT.

**Hippocampal ATP was reduced by TPP⁺ but unaltered by CsA.** Inhibition of the MPT by CsA may perturb the mitochondrial proton gradient important in ATP production (20). To address the possibility that CsA may alter hippocampal ATP concentrations, we measured total ATP concentration in hippocampal slices in the presence and absence of 10 µM CsA and found no significant change in ATP concentration caused by 10 µM CsA (Figure 5). To induce synaptic calcium influx as might occur during synaptic activity, hippocampal slices were exposed to 50 mM KCl for 2-10 minutes and then harvested for ATP quantification. Interestingly, ATP levels dropped to the same extent after KCl depolarization in both CsA-exposed slices and controls, and stay constant throughout KCl exposure (Figure 5A). Thus, CsA did not affect ATP concentration before or after KCl depolarization, which suggests that changes in synaptic function caused by CsA are not due to altered ATP availability.

Sustained exposure to 100 µM TPP⁺ caused a significant declined in levels of ATP in the hippocampal slice after 40 minutes of incubation (Figure 5B). The decline in ATP correlated with the degradation of electrophysiological signals in hippocampal slices continuously exposed to 100 µM TPP⁺ (see Figure 2D). In
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contrast, hippocampal slices exposed to 10 µM CsA did not show altered ATP levels and accordingly maintained electrophysiological integrity (Figure 5B).

Effects of CsA on presynaptic calcium. The effect of CsA on synaptic function suggested a two-phase mechanism, an immediate increase in baseline synaptic transmission followed approximately 20 minutes later by an attenuation in synaptic plasticity (see Figure 2). We hypothesized that the changes in synaptic function might be due to secondary changes in mitochondrial calcium handling. In order to determine the effect of CsA on calcium handling, we monitored cytosolic calcium within pre-synaptic terminals exposed to CsA in acute hippocampal slices using an adaptation of our previously published protocol (8). Briefly, the calcium indicator FURA-2 was applied to the axons of the Schaffer-collateral (SC) pathway and transported to pre-synaptic terminals in CA1. This allowed us to measure relative changes in fluorescence caused by calcium binding to FURA-2 (Figure 6A).

Using this approach, we were able to monitor the real-time effects of CsA on pre-synaptic calcium. Immediately upon exposure to 10 µM CsA, resting pre-synaptic calcium was rapidly increased, followed by a more gradual return to baseline by 30 minutes (Figure 6B). Application of the vehicle, ethanol, had no effect (Figure 6C). Thus, the increase in baseline synaptic transmission that we observed in Figure 2B correlates with a transient increase in resting calcium over
the same time period. Interestingly, the return of resting calcium to baseline levels occurs at about the same time that the PPF deficit began (see Figure 3).

To confirm the target of CsA on mitochondrial calcium stores, we performed the same experiment using TPP$^+$ to depolarize mitochondria. By depolarizing mitochondria, we effectively remove the ability of mitochondria to take up calcium. As expected, exposure to 100 µM TPP$^+$ immediately caused mitochondrial depolarization and consequent calcium release (Figure 6D) due to loss of the mitochondrial proton gradient. When CsA was added to the TPP$^+$ bath 30 minutes later, there was no effect (Figure 6D), suggesting that the target of CsA and TPP$^+$ are the same, i.e., mitochondrial calcium stores. When the TPP$^+$ was washed out for 10 minutes, mitochondria recovered and CsA regained its ability to cause an increase in resting calcium (Figure 6E).

CsA did not measurably affect stimulus-evoked calcium transients using the paired-pulse protocol (Figure 6F) suggesting that rather than participating in the dynamic handling of calcium during paired-pulse stimulation, the MPT is more involved in regulating resting pre-synaptic calcium concentration. Thus, we can now correlate changes in resting pre-synaptic calcium handling with alterations in synaptic function in hippocampal slices exposed to CsA.

Surprisingly, the effect of TPP$^+$ on resting pre-synaptic calcium remarkably resembled the effect of CsA (Figure 6E), again suggesting a common
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mechanism. As TPP$^+$ is known to depolarize mitochondria as part of its mechanism of causing calcium release, we used an *in vitro* assay of calcium handling in isolated mitochondria to determine if CsA might be affecting mitochondrial calcium handling as well.

**Calcium buffering in isolated mitochondria is altered by CsA.** Intact and functional isolated brain mitochondria rapidly buffered extramitochondrial calcium via the mitochondrial calcium uniporter, which could be blocked by ruthenium red (RuR) (Figure 7A). Calcium influx is driven by a respiration-dependent electrochemical gradient across the mitochondrial inner membrane. Carbonyl cyanide 3-chlorophenylhydrazone (CCCP), which dissipates the mitochondrial electrochemical gradient, also blocked calcium influx and, in addition, induced mitochondrial depolarization and subsequent calcium release when added at the point of maximal buffering (Figure 7A). Overwhelmed by the amount of calcium used in this *in vitro* assay, control mitochondria eventually depolarize and subsequently release their stores of calcium. These observations are consistent with previously published studies on mitochondrial calcium handling (21) and indicate that our isolated mitochondrial preparation contains intact and functional mitochondria capable of sequestering calcium.

Exposure of mitochondria to CsA prior to a 5-10 µM calcium bolus resulted in an increased rate of calcium influx (Figure 7B) and an earlier and more rapid mitochondrial depolarization and subsequent calcium release (Figure 7C). CsA
exposure to at least three different mitochondrial preparations consistently resulted in an increased influx rate and more rapid calcium release (1.6-2.0 fold faster influx and release in each preparation), and consequently greater extra-
mitochondrial calcium. Thus, the transient increase in cytosolic calcium observed in pre-synaptic terminals exposed to CsA (see Figure 6B) may have been due to triggering of mitochondrial depolarization and subsequent calcium release.

Mitochondrial depolarization by CsA is not a direct effect, as with TPP$^+$ and CCCP, because CsA added after maximal calcium buffering did not induce mitochondrial depolarization and subsequent calcium release (Figure 7D). In order for CsA to depolarize mitochondria, it had to be present before calcium influx. One interesting possibility is that the increased rate of calcium influx caused by CsA triggered an earlier and more rapid mitochondrial depolarization and subsequent calcium release.

**Role of VDACs in Calcium Buffering in Isolated Mitochondria.** We interpret the effect of CsA on mitochondrial calcium handling as being due to inhibition of the MPT. To confirm this interpretation, we evaluated the effects of genetic deletion of an outer pore component of the MPT, VDAC1, using the *in vitro* method above. Similar to CsA-exposed wild-type mitochondria, VDAC1-deficient mitochondria depolarized and released calcium more rapidly compared to littermate controls (Figure 7E), which ultimately resulted in greater extra-


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mitochondrial calcium. This common feature of mitochondrial depolarization and increased extra-mitochondrial calcium may explain the electrophysiological similarity of CsA exposed wild-type hippocampal slices and VDAC-deficient hippocampal slices in synaptic plasticity (4).

DISCUSSION

Our data along with other recent reports suggest that mitochondrial calcium buffering is an important regulator of synaptic function (22,23). Extracellular field recordings in hippocampal slices exposed to low doses of CsA, which functionally inhibits the mitochondrial permeability transition pore, showed significant deficits in PPF and 100 Hz LTP and an increase in baseline synaptic transmission. Calcium imaging of presynaptic terminals loaded with FURA-2 demonstrated that CsA induces an increase in resting calcium that may account for the acute change in synaptic transmission. While the changes in presynaptic calcium and baseline synaptic transmission were acute effects of CsA, the deficits in short-term synaptic plasticity began as the resting presynaptic calcium returned to baseline, approximately 20 minutes after CsA exposure. This delayed outcome of the synaptic plasticity deficit might be due to a secondary effect of the transient calcium increase or to prolonged mitochondrial depolarization. The former possibility is supported by previous studies that show that elevated presynaptic calcium can activate calcium-calmodulin kinases that phosphorylate the vesicle associated proteins synapsins; synapsin
phosphorylation may result in impaired presynaptic plasticity (17). However, as indicated in Figure 4, 10 µM CsA exposure does not result in phosphorylation of synapsin I at any of the sites tested suggesting that synapsin phosphorylation is not likely mediating the changes in synaptic plasticity caused by CsA. Nevertheless, there are numerous other calcium-sensitive pathways within synaptic terminals that may impinge on mechanisms important in synaptic plasticity. Alternatively, the transient increase in calcium may represent a more generalized mitochondrial malfunction resulting from prolonged depolarization. In support of this idea are recent studies that show that respiratory chain uncouplers affecting mitochondrial calcium handling disrupt synaptic plasticity in the Calyx of Held (23) and the crayfish neuromuscular junction (22).

The location of mitochondria in pre-synaptic terminals does not lend itself well to buffering calcium influx from the extracellular space during single synaptic depolarizations (24) as this type of calcium influx may cause calcium elevations restricted to a smaller submembranous space. Rather, mitochondria appear to be more important in regulating the overall resting calcium level. In order to understand the role of the MPT and VDACs in regulating the resting calcium level, we employed an in vitro assay of mitochondrial calcium buffering. Studies of calcium buffering in isolated mitochondria are useful for understanding the roles of channels, exchangers and pores in calcium influx and/or efflux, but their behavior in vivo might differ from their function in vitro. For example, although a calcium bolus of low micromolar concentration is necessary to observe the effect
of CsA in isolated mitochondria, resting calcium levels in pre-synaptic terminals do not likely reach these levels. In addition, isolated mitochondria include those from both pre-synaptic and post-synaptic compartments as well as glia. For these reasons, this assay only serves as a model for the role of the MPT in calcium regulation which might help to explain the function of mitochondrial buffering in the intact pre-synaptic terminal in synaptic transmission and plasticity.

It appears that \textit{in vitro} mitochondrial calcium handling can be functionally divided into two stages, the uptake phase and the release phase. The uptake phase is dominated by calcium uptake by the uniporter. Blocking the MPT with CsA resulted in an increased net uptake during this phase. There are two workable hypotheses to explain this effect of CsA. First, if CsA hyperpolarizes the mitochondrial membrane, the drive for calcium influx would be greater and would thus cause increased calcium influx. Alternatively, one could suppose that the MPT conducts calcium release and, if blocked by CsA, would lead to a greater net influx. Either mechanism is plausible to explain the results of this assay but more importantly, the upshot is the same, that is, excessive calcium influx caused by blockage of the MPT results in calcium overload and subsequent mitochondrial depolarization. The result of mitochondrial depolarization is rapid calcium release from isolated mitochondria, as observed with CCCP. Consistent with this mechanism, adding CsA at the point of maximal calcium buffering did
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not have any effect (Figure 59), indicating that the effect of CsA is dependent on calcium influx specifically.

It is not yet known how calcium exits the mitochondria during the release phase but based on our studies, it is not likely to be via the MPT or the Na\(^+/\)Ca\(^{2+}\) exchanger (data not shown) as inhibitors of these pathways did not affect calcium release during this phase. The trigger for calcium release appears to be mitochondrial depolarization by cation overload. While there is a correlation between increasing the rate of calcium uptake, by adding CsA, and inducing earlier calcium release, mitochondria can buffer anywhere from 5-75 µM total calcium before releasing it. Thus, it is not the total calcium uptake, but the rate of calcium uptake, that appears to play a role in triggering calcium release in our in vitro preparation.

The increase in resting calcium stimulated by CsA strongly resembles the increase stimulated by TPP\(^+\) suggesting at least a common locus of action and possibility a common mechanism of action in the pre-synaptic terminal. TPP\(^+\), a lipophilic cation works by carrying its positive charge across mitochondrial membranes into the negatively charged matrix and depolarizing it by virtue of overloading the matrix with positive charges. CsA is believed to work by binding to cyclophilin D, the matrix component of the MPT, and functionally blocking the pore. The in vitro assay results suggest that blockage of the MPT leads to calcium (or other cation) overload and subsequent mitochondrial depolarization,
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complete with rapid calcium release. Thus, we can now speculate why CsA and TPP⁺ have the same effect on resting pre-synaptic calcium. They both cause mitochondrial depolarization leading to calcium release: TPP⁺ does it by virtue of loading positive charges into the matrix while CsA does it by overloading the matrix with calcium, or other cations such as potassium, sodium and/or protons. Mitochondria lacking VDAC1 were also more likely to depolarize and subsequent release their calcium stores. VDAC1-deficient mitochondria may be more vulnerable to calcium overload resulting in more rapid calcium release as observed in in vitro assays of VDAC1-deficient isolated mitochondria.

While previously known mitochondrial calcium-specific transporters and channels have only been observed on the mitochondrial inner membrane, the mechanism of calcium transport across the mitochondrial outer membrane is not known. VDACs are the only known channels that may be able to conduct calcium into the intermitochondrial space (25) and therefore may play an important function in calcium influx into the mitochondrial matrix. Although calcium flux into the mitochondrial matrix has hitherto been attributed solely to the calcium uniporter of the mitochondrial inner membrane, we think it is interesting that VDAC-1 deficient mitochondria were impaired in mitochondrial calcium uptake in this assay (Figure 7F). This finding is consistent with the growing notion that the mitochondrial outer membrane is not simply a molecular sieve, but rather a regulated barrier that is involved in calcium flux into the intermitochondrial space, the source of calcium for the uniporter. Thus, the role of VDACs in calcium flux
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may not only be as a component of the MPT but also as calcium regulators across the mitochondrial outer membrane.

Putting all the clues together, our study defines the roles of VDACs and the MPT in synaptic function. Based on the in vitro assay results, the MPT and VDACs serve to regulate the release of cations from mitochondria to prevent overload and subsequent depolarization. VDAC-deficiency is detrimental for the proper function of the MPT as absence of VDAC1 resulted in an increased likelihood of mitochondrial depolarization and subsequent calcium release as well. The overall result of mitochondrial depolarization in pre-synaptic terminals, induced by TPP⁺, CsA or absence of VDACs is dysregulation of resting calcium which may impinge on signal transduction pathways important in synaptic transmission and plasticity.
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Acknowledgements: This work was supported by NRSA grant (ML) F31NS42488 and NIH grants (JDS) MH57014 and HD24064.
Figure Legends

Figure 1
Electron micrograph of hippocampus area CA1. In this EM of a rat hippocampus area CA1 (previous published (26), permission by Harris, KM), mitochondria can be seen packed into pre-synaptic terminals (grey arrows) and dendrites (black arrows).

Figure 2
CsA impaired LTP and enhanced synaptic transmission. A. CsA exposure for 20 minutes before HFS resulted in impaired long term potentiation induced by 100 Hz stimulation (open squares=control, closed squares=CsA, n=6 for both). The average potentiation in the presence of the CsA was 146 ± 0.15% of baseline versus in control where the average potentiation was 190 ± 0.31%. Traces represent control (black) and CsA-exposed (grey) fEPSPs before (top) and after (bottom) high frequency stimulation, vertical scale = 0.2mV, horizontal scale = 2 ms. B. Baseline synaptic transmission increased almost immediately upon exposure to CsA by approximately 25% (open squares=control, closed squares=CsA, n=3 each, p=0.02). C. Theta-burst induced LTP was not attenuated by CsA (open squares = control, closed squares = CsA, normalized to last 10 minutes of baseline fEPSPs, n=6 for both.) Traces represent control (black) and CsA-exposed (grey) fEPSPs before (top) and after (bottom) high frequency stimulation, vertical scale = 0.2mV, horizontal scale = 2 ms. D. 100 µM TPP+ added 20 minutes prior to a 100 Hz LTP stimulation paradigm resulted in attenuated early LTP, somewhat similar to the effect of CsA on LTP (dashed line=control, solid line=CsA, TPP+=open circles) followed by a degradation of signal after 1 hour of recording.

Figure 3
CsA attenuated PPF. A. A second effect of CsA, apparent 20 minutes after CsA-exposure, was an attenuation in PPF (n=9) compared to controls (n=8) in (B). C. The average change in facilitation beginning 30 minutes after CsA exposure until the end of recording at 90 minutes post-exposure was attenuated by approximately 4% (n=9) as compared to controls (n=8), p<0.0001.

Figure 4
Calcineurin Inhibition by High Doses vs. Low Doses of CsA. Synapsin I phosphorylation can be used to assess calcineurin inhibition in hippocampal slices. Ser603, Ser553, and Ser62/67 showed enhanced phosphorylation when incubated with 250 µM CsA as compared to 10 µM or no CsA indicating that low doses of CsA (10 µM) were not inhibiting calcineurin.

Figure 5
TPP+, but not CsA, reduced ATP levels in hippocampal slices. A. Hippocampal slices exposed to CsA did not show changes in ATP concentration (no KCl), n=9, p=0.442. Incubation in 50 mM KCl, to induce presynaptic calcium
influx, with or without 10 \( \mu \text{M} \) CsA, showed a decrease in ATP content of equal extent 2 minutes following depolarization that stayed constant throughout the 10 minute period of KCl exposure. Thus CsA did affect ATP concentration before or after KCl depolarization. B. Sustained exposure to TPP+ over 40 minutes caused a significant decline in ATP levels as compared to control and CsA-exposed slices (n=7 each, p < 0.02).

Figure 6
Pre-synaptic calcium alterations by CsA. A. Calcium imaging of presynaptic terminals was performed by injecting the calcium indicator FURA-2 into axons of the Schaffer-collateral (SC) pathway. The FURA-2 was transported to the pre-synaptic terminals in CA1 where we measured relative changes in fluorescence caused by calcium binding to FURA-2. Stimulus-evoked calcium transients were measured by stimulating axons of the SC pathway. B. CsA caused an acute and transient increase in background calcium, detected as an increase in fluorescence ratio (340nm/380nm), that returned to baseline in 30 minutes as compared to control in (C) (n=7 for each). D. Prior depolarization by TPP+ abolished the effect of CsA. E. After washing TPP+ out for 10 minutes, CsA regained its ability to cause an immediate and transient increase in resting calcium. F. Stimulus-evoked calcium was not affected by CsA. Data points represent change in amplitude of fluorescence during the first EPSP (n=5, sample traces of stimulus-evoked calcium influx: black=before CsA, grey=after CsA exposure).

Figure 7
The MPT is involved in mitochondrial calcium handling. A. When exposed to a bolus of free calcium, intact functional mitochondria rapidly absorbed the calcium. Calcium overload eventually caused mitochondrial depolarization and subsequent calcium release. Calcium influx is mediated by the uniporter which could be blocked by RuR and is dependent on an intact mitochondrial proton gradient which could be uncoupled with CCCP (added at the arrow after maximal calcium buffering). B. Blocking the MPT with CsA resulted in a significantly more rapid calcium influx (n=4 each). C. Increased influx by CsA also led to a more rapid mitochondrial depolarization and subsequent calcium release (n=4 each, difference in extramitochondrial calcium at 6 min p=0.0007, rate of efflux (\( \mu \text{M/sec} \)): control=0.86±0.04, CsA=1.51±0.06). D. When CsA was added at the point of maximal buffering (added at the arrow), there was no change in calcium uptake or efflux (p<0.001 for all time points < 2 min). E. VDAC1-deficient mitochondria depolarized and subsequently released calcium faster compared to wild-type controls resulting in significantly more extramitochondrial calcium during the efflux phase (n=3 mito preps., 4 samples from each prep., difference in extramitochondrial calcium at 10 min p=0.013, rate of efflux (\( \mu \text{M/sec} \)): wild-type=0.45±0.08, mutant=0.80±0.24). F. VDAC1-deficient mitochondria were impaired in their ability to buffer calcium as much as littermate controls (n=3 mito preps, 4 samples from each prep.)
Figure 2

A

B

C

D

Mitochondria in synaptic plasticity
Figure 3

A

% Change in Facilitation

Cyclosporin A

Time (min)

B

% Change in Facilitation

EtOH vehicle

Time (min)

C

% Change in Paired-Pulse Facilitation

CsA  Control

***
Mitochondria in synaptic plasticity

Figure 4
Figure 5

A

![Bar graph showing nmoles ATP/mg protein with error bars for Control and 10μM CsA treatments under different conditions: No KCl, KCl (2 min), KCl (10 min).]

B

![Bar graph showing Relative ATP/protein for Control, CsA, and TPP* treatments.]

Mitochondria in synaptic plasticity
Figure 6

A

Stimulation electrode
Injection of Fura-2 AM
Optical recording area

C

Percent Change in Fura-2 Ratio (%)

Time (min)

EtOH vehicle

E

% Change in Fluorescence ([340/380])

Time (min)

TPP+ CsA

B

Percent Change in Fura-2 Ratio (%)

Time (min)

Cyclosporin A

D

% Change in Fluorescence ([340/380])

Time (min)

TPP+ CsA

F

% Change in Fluorescence (amplitude of calcium influx)

Time (min)

Cyclosporin A
Figure 7

A  
Free calcium (μM)  
Time (min)  
Control  
RuR  
CCCP

B  
Free calcium (μM)  
Time (min)  
Control  
CsA

C  
Free calcium (μM)  
Time (min)  
Control  
CsA

D  
Free calcium (μM)  
Time (min)  
Control  
CsA

E  
Free calcium (μM)  
Time (min)  
VDAC1 (+/+)  
VDAC1 (-/-)

F  
Free calcium (μM)  
Time (min)  
VDAC1(+/+)  
VDAC1(-/-)