[Ca$^{2+}$], Signaling between Mitochondria and Endoplasmic Reticulum in Neurons Is Regulated by Microtubules

FROM MITOCHONDRIAL PERMEABILITY TRANSITION PORE TO Ca$^{2+}$-INDUCED Ca$^{2+}$ RELEASE*

Sergej L. Mironov†, Maxim V. Ivannikov, and Mattias Johansson
From the Institute for Physiology, Georg August University, Humboldallee 23, 37073 Göttingen, Germany

The positioning and dynamics of organelles depend on membrane-cytoskeleton interactions. Mitochondria relocate along microtubules (MT), but it is not clear whether MT have direct effects on mitochondrial function. Using two-photon microscopy and the mitochondrial fluorescent dyes rhodamine 123 and Rhod-2, we showed that Taxol and nocodazole, which correspondingly stabilize and disrupt MT, decreased potential and Ca$^{2+}$ in the mitochondria of brain stem pre-Bötzinger complex neurons. Both effects were inhibited by pretreatment with blockers of mitochondrial permeability transition pore (mPTP), cyclosporin A, and 2-aminoethoxydiphenyl borate. Induction of mPTP by Taxol and nocodazole was confirmed by using a calcein/Co$^{2+}$ imaging technique. Electron and optical microscopy revealed tubulin bound to mitochondria. Mitochondria, MT, and endoplasmic reticulum (ER) showed strong co-localization, the degree of which decreased after MT were disrupted.

We propose that changes in the structure of MT by Taxol and nocodazole promote the opening of mPTP with subsequent initiation of Ca$^{2+}$ release from the ER (21, 24). In neurons, the positions of mitochondria and ER also showed a close positioning of mitochondria to the ER as determined by MT can be essential for the local [Ca$^{2+}$] signaling in neurons.

Interactions between intracellular membranes and microtubules (MT)† determine the structure and positioning of subcellular organelles and direct their dynamic movements. MT can associate with the organelles either dynamically or in a stable fashion. Dynamic interactions are required for organelle traffic and involve microtubule motors such as kinesin and dynein (1, 2). Stable interactions are responsible for the positioning and structural maintenance of the endoplasmic reticulum (ER) (3), and mitochondria (4–9). However, it is not known whether MT can influence the function of mitochondria and ER. Taxol (paclitaxel) and nocodazole, which correspondingly stabilize and disrupt MT, change the structure of the cytoskeleton. In proliferating cells, these drugs prevent normal mitotic spindle formation and cause the cells to halt mitosis and to initiate apoptosis. In comparison with MT-disrupting drugs, Taxol is less toxic and has recently been promoted for the treatment of ovarian, breast, lung, and prostate cancers (10), but it is often accompanied by serious peripheral neuropathies (11), the origin and mechanisms of which are yet unclear. Only one intracellular target has been established for Taxol thus far; it binds to a site located on the inner surface of the MT wall (12) and enhances lateral contacts between tubulin dimers (13). Recent evidence obtained in non-neuronal cells (14, 15) suggests that Taxol can directly target tubulin bound to mitochondria.

We show here that Taxol and nocodazole depolarized mitochondria and released previously stored Ca$^{2+}$ in brain stem pre-Bötzinger complex neurons. Both effects were inhibited by cyclosporin A (CsA) and 2-aminoethoxydiphenyl borate (ABP), commonly used blockers of mitochondrial permeability transition pore (mPTP). mPTP opening was validated by using the mPTP-specific calcein/Co$^{2+}$ imaging technique (16). The effects of Taxol were not mediated by enhanced Ca$^{2+}$ influx, leading to subsequent overload of mitochondria with Ca$^{2+}$ or overproduction of reactive oxygen species (ROS), the two main factors that can promote the opening of mPTP with subsequent initiation of apoptosis (17–19).

Electron and optical microscopy revealed close interactions of MT with mitochondria in living neurons and isolated mitochondria. We therefore suggest that mPTP induction is triggered by modification of MT interactions with proteins of the outer mitochondrial membrane, e.g. the voltage-dependent anion channel (VDAC) (9, 20–23). In non-neuronal cells mitochondria and ER are closely opposed, and mitochondria can capture Ca$^{2+}$ released from the ER (21, 24). In neurons, the positions of mitochondria and ER also showed a strong overlap, which disappeared after disruption of MT. Therefore we suggest that a close positioning of mitochondria and ER allows Ca$^{2+}$ released via mPTP to activate a Ca$^{2+}$-induced Ca$^{2+}$ release (CICR) from the ER but that this pathway is activated only when MT are intact and keep the two organelles in contact.
EXPERIMENTAL PROCEDURES

Cell Isolation and Culture—The use and care of animals in this study complied with the guidelines of the European Commission (No. L358, ISSN 0378-6978) and the Committee for Animal Research, Göttingen University. The procedures for isolation of the respiratory center have been described in full previously (25) and are mentioned briefly below.

Neonatal NMRI mice (P3–7) of both sexes were anesthetized with ether and decapitated at the C3–C4 spinal level. The preparation was made in ice-cold artificial cerebrospinal fluid containing (in mM): 136 NaCl, 5 KCl, 1.25 CaCl2, 0.8 MgSO4, 0.4 NaH2PO4, 0.3 K2HPO4, 3.3 NaHCO3, 6 glucose, pH 7.4, after equilibration with 95% O2, 5% CO2. Artificial cerebrospinal fluid was also used as a bathing medium in all experiments after isolating the brain stem, a 1.2–1.5-mm slice was cut, and patches containing the two symmetrically placed pre-Bötzinger complexes (26) were isolated by sucking the tissue into a large bore pipette. The pieces of tissue were subsequently incubated in 50 μl of Ca2+/Mg2+-free medium (containing 0.05% trypsin and 0.53 mM EDTA) at 37 °C for 10 min. The enzymatic reaction was stopped by the addition of 100 μl of high glucose Dulbecco’s modified Eagle’s medium containing additionally 15% horse serum, 2 mM l-glutamine, 10 μg/ml (10 units/ml) penicillin/streptomycin, 1 mM Na2-pyruvate. The cell suspension, obtained by gentle pipetting, was placed onto coverslips coated with poly-lysine. After the cells were allowed to sediment for 30 min at 37 °C, the incubation solution was changed to 500 μl of neurobasal-A medium, also containing B27 supplement, 0.5 mM l-glutamine, 10 μg/ml penicillin/streptomycin, and growth factors neurotrophin-3 (0.5 ng/ml) and brain-derived neurotrophic factor (BDNF, 0.5 ng/ml). The culture medium was changed on the next day after preparation and then every fourth day. The cells were used 4–8 days after preparation. For the experiments, the coverslips were mounted onto a glass slide glued to the bottom of the perfusion camera, and the cells were examined under a microscope. experiments were performed at 34 °C. The solution exchange was achieved by switching between two reservoirs, and it took about 20 s to completely exchange the chamber contents.

Cell Staining and Fluorescence Measurements—The cells were incubated at 37 °C in the dark with 2 μg/ml rhodamine 123 (Rh-123) for 25 min, with 1 μM fluo-3/AM for 20 min, 1 μM calcine/AM for 30 min (16), or with 5 μM rhod-2/AM for 1 h. After loading, the cells were kept in a dye-free medium for at least 1 h before the experiments began. Cells were transfected with cDNA encoding enhanced yellow or cyan fluorescent tubulin or actin (Clontech, Heidelberg). 6 μl of solution containing 1 μg of cDNA and 2.7 μl of FuGENE 6 (Roche Applied Science) was added to 97.3 μl of neurobasal-A medium without complement and thoroughly mixed with 400 μl of fresh neurobasal-A medium for 15 min. The transfection efficiency was ±12%.

The images were obtained by using either (a) a two-photon scanning microscope (performance described in full elsewhere (27)) or (b) a cooled charge-coupled device camera (CCD, MicroMax, Princeton Instruments) or (c) a confocal microscope (Zeiss Axioskope, and the cells were viewed under ×63 objectives (Achroplan, N.A. 0.95). Images were digitalized at 8- (a) or 12-bit (b) resolution and were collected by using custom-made (a) or Meta Morph (b, c) software. All experiments with one indicator dye were made by using a two-photon scanning microscope. The illumination wavelength was set to 800 nm, and the pixel acquisition time was 5–1 μs. Digital images were acquired every 5–7 s, and the average fluorescence intensity of all relevant regions was recorded and stored for a subsequent analysis. The images were analyzed by using Meta Morph software (Meta Imaging series, Universal Imaging Corp.).

For simultaneous measurements of fluorescence of two indicator dyes, a monochromator light source was used (Till Photonics, Planegg, Germany). Images of wide field fluorescence were collected with exposure times ranging between 100 and 300 ms. Yellow fluorescent protein (GFP), Flutax-1, rhod-2, fluo-3, calcine, and Rh-123 were excited at 490 nm, and the fluorescence was isolated by using a dichroic mirror with mid-reflection at 505 nm and an emission filter (535 ± 15 nm). For monitoring pECFP fluorescence, the cells were excited at 435 nm by using a dichroic mirror with mid-reflection at 480 nm, and cyan fluorescence was additionally filtered by using an emission filter (475 ± 20 nm). Mitochondria and ER were colocalized with MitoTracker and ER-Tracker, respectively, both applied at 1 μM for 30 min at 37 °C. The dyes were excited at 380 nm (ER-Tracker) and 480 nm (MitoGreen-Tracker), and the fluorescence was collected by using an emission filter (535 ± 15 nm). The degree of overlap between mitochondria and ER was calculated from the ratio of areas occupied by common pixels in the superimposed image and that of ER.

Potentiometric dyes are widely used to assess Ψm changes in different preparations under various conditions (28–33). The patterns of rhod-2 and Rh-123 fluorescence in neurons showed punctate structures representing individual mitochondria (Fig. 1). The fluorescence was measured in 2-μm-diameter regions of interest encircling the bright spots corresponding to mitochondria. The changes in the fluorescence of Rh-123 measured under different experimental conditions are in line with changes in Ψm expected from known mitochondrial properties (17, 18). The signals of 4–6 mitochondria within a cell were averaged, and the changes in fluorescence are presented as ΔF/F0 ratios (ΔF = F – F0), where F is the measured fluorescence and F0 is the initial fluorescence. Before and after each trial, the cell morphology was observed in transmitted light to ensure that experimentally induced cell death had not occurred. Each test was repeated for at least three different cell preparations, and means ± S.E. were compared by using Student’s t test, with p < 0.05 being the condition for statistical significance. Analysis of variance was used for multiple comparisons.

In fluo-3-loaded cells the staining was diffuse, and the signals obtained from different cell regions showed similar time dependencies. The exceptions were the local and propagating [Ca2+]i transients in

FIG. 1. Mitochondria, tubulin, and [Ca2+]i in pre-Bötzinger complex neurons. Shown are the difference interference contrast (DIC) transmittance (A), rhod-2 (B), Flutax-1 (C), Rh-123 (D), and fluo-3 (E) images obtained as described under “Experimental Procedures.” Scale bar = 10 μm. D and E, Taxol-induced changes in mitochondrial potential and [Ca2+]i. The local peaks in frames taken at 5-s intervals are connected by white lines to show the progression of signals from dendrites toward soma.
dendrites initiated by Taxol (Fig. 1E). The functional viability of fluo-3-loaded neurons was routinely tested by using brief (5 s) applications of 45 mM KCl. This increased the signal of fluo-3 by about 3-fold, corresponding to the elevation of \([Ca^{2+}]_i\) to 0.6 mM. This test was consistently used to verify that neurons are at proper resting potential and possess both functional voltage-sensitive \([Ca^{2+}]_i\) clearance mechanisms (34).

It has been reported that the extent of cell and hence mitochondrial loading with potentiometric probes can be affected by the activity of the plasma membrane multi-drug resistance P-glycoprotein, which is inhibited by CsA (16). Therefore in the experiments using CsA as a blocker of mPTP, the drug was added to the cells at least 1 h after the dyes were loaded. Fluorescence acquisition started 30 min after CsA addition, a protocol that excludes any effects that may be mediated by multi-drug resistance. In addition, some experiments were performed by supplementing artificial cerebrospinal fluid with 1.6 mM CsH, which inhibits the multi-drug resistance pump but not the mPTP (16). No difference in the results was noticed.

**Materials**—Nifedipine, CsA, Taxol (paclitaxel), APB, poly-D-lysine, L-glutamine, and horse serum were purchased from Sigma. Oligomycin,
Thapsigargin, and Flutax-1 were from Calbiochem. Rh-123, rhod-2/AM, fura FF/AM, calcine/AM, and MitoGreen- and ER-Tracker were from Molecular Probes (MolBiTec, Göttingen, Germany). Dulbecco’s modified Eagle’s medium, Trypsin/EDTA solution, neurobasal-A medium, B27 supplement, and penicillin/streptomycin were from Invitrogen. All chemicals were reagent grade or higher. Rh-123 and oligomycin were dissolved in absolute ethanol. Other dyes and drugs were prepared as stock solutions in Me2SO.

**Estimation of Acting Concentration of Taxol**—The concentration dependence of the effects of Taxol was quantified by using the fluorescent probes MitoTracker Green and MitoTracker Red. Mitochondria were loaded with MitoTracker Green and MitoTracker Red for 10 min and subsequently stained with Rh-123 and Fura FF. Mitochondria were loaded with 1 μM Fura FF/AM for 20 min at 37 °C, and the fluorescence was recorded at excitation/emission wavelengths of 380 and 520 nm, respectively. Mitochondria were functionally active up to 10 h after isolation, and they retained both their membrane potential and Ca2⁺ depolarization.

**Isolation of Brain Mitochondria and Immunostaining of Tubulin**—Brain mitochondria were isolated from P3-P7 mice. All steps during mitochondria isolation were performed at 4 °C. Before homogenization, 40 mg of brain tissue was washed with 1 ml of extraction buffer consisting of 200 mM mannitol, 70 mM sucrose, 1 mM EGTA, and 10 mM HEPES, pH 7.5. Brain homogenate was obtained by gentle pipetting in ice-cold extraction buffer containing 2 mg/ml bovine serum albumin and centrifuged at 1000 × g for 10 min, and the resulting pellet was dissolved in 1 ml of extraction buffer and centrifuged at 1000 × g for 5 min. The supernatant was centrifuged at 3000 × g for 10 min, and the resulting pellet was dissolved in 1 ml of extraction buffer and centrifuged at 10000 × g for 10 min. The final pellet, representing the mitochondrial fraction, was dissolved in 0.2 ml of storage buffer consisting of 250 mM sucrose, 1 mM ATP, 0.08 mM ADP, 5 mM sodium succinate, 2 mM K2HPO4, 1 mM dithiothreitol, and 10 mM HEPES, pH 7.4. Isolated mitochondria were immobilized on glass coverslips by centrifugation at 2000 × g for 10 min and subsequently stained with Rh-123 and fura FF. Mitochondria were loaded with 1 μM Fura FF/AM for 20 min at 37 °C, and the fluorescence was recorded at excitation/emission wavelengths of 380 and 520 nm, respectively. Mitochondria were functionally active up to 10 h after isolation, and they retained both their membrane potential and Ca2⁺ depolarization.

**RESULTS**

**Taxol and Nocodazole Induce mPTP**—Application of Taxol (10 μM) produced immediate mitochondrial depolarization (Fig. 2A). In addition, in 6 of 12 cells examined, Taxol also induced
spontaneous $\Psi_m$ changes. These transients propagated from dendrites toward soma and resembled the $[\text{Ca}^{2+}]_i$ waves induced by Taxol (Fig. 1D). Pretreatment with 15 $\mu$m cyclosporin A for 30 min prior to the addition of Taxol abolished any $\Psi_m$ changes (Fig. 2A). This suggests that the effects of Taxol on mitochondria are caused by the opening of mPTP. In isolated brain mitochondria, Taxol depolarized mitochondria (Fig. 2C) and released previously stored $\text{Ca}^{2+}$ (for $n = 4$, data not shown). Both effects were inhibited by CsA.

Given the effects of Taxol, it was interesting to examine the effects of nocodazole, which has an opposite action on MT. Nocodazole (10 $\mu$m) depolarized neuronal mitochondria, but its effects were weaker, developed more slowly, and were not fully inhibited by CsA (Fig. 2B). Isolated brain mitochondria were rapidly (within 1 min) depolarized by nocodazole; this effect was prevented by CsA (Fig. 2C), and the actions of Taxol and nocodazole were mutually exclusive. This difference in the results obtained in neuronal and isolated mitochondria was probably caused by the slow permeation of nocodazole across the plasma membrane or by other factors (38, 39). We therefore tested another mPTP blocker, 2-aminooxydiphenyl borate (39). APB completely abolished changes in $\Psi_m$ induced by nocodazole (Fig. 2) and Taxol (for $n = 3$). Cytochalasin B, an actin-epolymerizing agent that modifies $[\text{Ca}^{2+}]_i$ signaling in hippocampal cells (40), had no effect on mitochondrial variables.

Induction of mPTP was next monitored by applying a calcein/Ca$^{2+}$ imaging technique. Taxol induced a loss of calcein from mitochondria, an effect that was prevented by pretreatment with 10 $\mu$m CsA (Fig. 3) and 100 $\mu$m APB (for $n = 3$). The application of nocodazole produced similar effects (for $n = 3$, data not shown).

A variety of factors can promote the opening of mPTP; the most important of them are ROS (41) and $[\text{Ca}^{2+}]_i$ (17, 18). In the presence of the radical scavengers L-cysteine and N,N'-diphenyl-1,4-phenylenediamine (both at 0.1 mM, for $n = 4$), depolarization of mitochondria by Taxol was unchanged, excluding overproduction of ROS under these conditions. Taxol depolarized mitochondria in Ca$^{2+}$-free solution (for $n = 3$), which speaks against the overloading of mitochondria with Ca$^{2+}$.

**MT-acting Drugs Change Cytosolic and Mitochondrial Ca$^{2+}$**—Both Taxol and nocodazole induced a slow release of mitochondrial Ca$^{2+}$, which was blocked after pretreatment with CsA and APB (Fig. 4, A–C). $[\text{Ca}^{2+}]_i$ and $[\text{Ca}^{2+}]_m$ changes produced by nocodazole had similar kinetics (Fig. 4, B and C); the half-times for $[\text{Ca}^{2+}]_i$ increase and $[\text{Ca}^{2+}]_m$ decrease were 2.9 ± 0.9 and 3.1 ± 0.3 min, respectively. Notably, Taxol did not change the basal $[\text{Ca}^{2+}]_i$ level, which also speaks against the role of mitochondrial Ca$^{2+}$ overload as a possible trigger of mPTP induction. In 40% of neurons (20 of 50 examined) Taxol induced spontaneous $[\text{Ca}^{2+}]_i$ elevations (Fig. 4, D and E), which were not observed in the presence of CsA or APB. Similar to $\Psi_m$ transients, $[\text{Ca}^{2+}]_i$ spikes appeared first in dendrites and then spread out toward the soma (Fig. 1E).

$[\text{Ca}^{2+}]_i$ spikes were abolished after the blockade of Ca$^{2+}$ influx with 0.1 mM Cd$^{2+}$ (Fig. 4D) and Ca$^{2+}$-free solution (Fig. 4E), possibly reflecting a requirement for Ca$^{2+}$ influx in the activation of the CICR mechanism in neurons (42, 43). Thapsigargin, a SERCA inhibitor, depleted the ER (34) as revealed by a slow $[\text{Ca}^{2+}]_i$ transient (Fig. 4F). The subsequent addition of Taxol produced a long lasting $[\text{Ca}^{2+}]_i$ increase. The empty ER and the weak effects of Taxol on the basal Ca$^{2+}$ influx points toward the mitochondria as a Ca$^{2+}$ source and the ER as a Ca$^{2+}$ store during mPTP activation. In the presence of CsA, thapsigargin evoked a similar $[\text{Ca}^{2+}]_i$ transient, but the subsequent addition of Taxol was without effect.

![Fig. 5. Colocalization of microtubules, mitochondria and endoplasmic reticulum.](http://www.jbc.org/)

**Taxol and nocodazole did not appreciably modify the depolarization-induced $[\text{Ca}^{2+}]_i$ transients, induced by the application of 45 mM KCl; their amplitude and the time constant of $[\text{Ca}^{2+}]_i$, recovery were respectively increased by 25 ± 5% and 23 ± 6% (for $n = 6$ for both drugs). As mitochondria become "leaky" after mPTP induction, these values indicate a contribution of the mitochondria to the mechanisms of Ca$^{2+}$ clearance from neuronal cytoplasm, which is similar to the estimates obtained in hippocampal cells (34). Weak changes in depolarization-induced $[\text{Ca}^{2+}]_i$, transients also rule out nonspecific effects of MT-acting drugs that might reflect a general compromise of neuronal function.**

**Close Contact between Mitochondria, ER, and Microtubules**—The alteration of mitochondrial function by Taxol and nocodazole suggests specific interactions between MT and mitochondria, and $[\text{Ca}^{2+}]_i$ changes induced by these drugs imply the active role of the ER. MT showed considerable overlap with mitochondria (Fig. 5A). Nocodazole induced the relocation of mitochondria in dendrites to the soma, but the pattern of ER distribution was not appreciably modified (Fig. 5C). After MT disruption, the degree of overlap between ER and mitochondria decreased from 30% in dendrites and 49% in the soma to 3 and 11%, respectively (Fig. 5C). Similar observations were made in cells co-transfected with CDNA encoding, correspondingly, cyan and yellow fluorescent protein constructs of the resident proteins, calreticulin and cytochrome oxidase. Tubulin fragments were found in the isolated single mitochondria (Fig. 5B).
pore is permeable only to small molecules, The opening of low conductance mPTP is reversible, and the local Ca\(^{2+}\) knowledge, the actions of nocodazole on mitochondria have effects began to develop 1 h after addition of the drug. To our increased respiration rate and ROS production, but these ef-

1,4,5-trisphosphate infusion (45). However, these effects can be explained as the modulation of the ER (not the mitochondrial) 1,4,5-trisphosphate infusion (45). However, these effects can be explained as the modulation of the ER (not the mitochondrial) system. MT are additionally needed to keep ER and mitochondria from mitochondria to ER and activation of the CICR mechanism. MT network plays an important and specific role in the main-

Interactions between MT and mitochondria are influenced by mechanical stress imposed by MT-acting drugs may induce conformational changes in tubulin structure (50), which can promote interaction between VDAC and the adenine nucleotide translocase, leading to a subsequent formation of mPTP.

The bulk of cellular tubulin is cytoplasmic, but a significant fraction is firmly associated with the plasma membrane and intracellular membranes (Refs. 3–9 and this study). For example, cyclic-nucleotide phosphodiesterase links MT to the plasma membrane (51), and p63 links MT to ER (52). Which proteins attach MT to mitochondria is presently unknown. Interactions between MT and mitochondria are influenced by ATP hydrolysis (8), implying that molecular motors, the force-
generating enzymes, produce sufficient strain to modify the structure of the outer mitochondrial membrane. However, in contrast to tubulin and VDAC, the motor proteins are not co-isolated with mitochondria (Ref. 9 and this study (Fig. 5E)).

In conclusion, our data provide functional evidence that the MT network plays an important and specific role in the maintenance of mitochondrial function by regulating mPTP opening and positioning of mitochondria close to the ER. Coordination of [Ca\(^{2+}\)]\(_i\), signaling between mitochondria and ER by MT can be important in the control of neuronal functions, including temporal integration of [Ca\(^{2+}\)]\(_i\), dynamics (53) and long term changes in [Ca\(^{2+}\)]\(_i\), that underlie synaptic plasticity (54). Further studies are needed to elucidate the role(s) that MTs play in local Ca\(^{2+}\) signals in neurons generated by Ca\(^{2+}\) exchange between mitochondria and ER.

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