Paclitaxel Affects Cytosolic Calcium Signals by Opening the Mitochondrial Permeability Transition Pore*

Received for publication, July 19, 2001, and in revised form, October 31, 2001
Published, JBC Papers in Press, November 27, 2001, DOI 10.1074/jbc.M106802200

Jackie F. Kidd, Mary F. Pilkington, Michael J. Schell‡, Kevin E. Fogarty§, Jeremy N. Skepper®,
Colin W. Taylor, and Peter Thorn¶

From the §Biomedical Imaging Group, Department of Physiology, University of Massachusetts Medical School, Worcester, Massachusetts, the ‡Department of Anatomy and the Department of Pharmacology, Cambridge University, Tennis Court Road, Cambridge CB2 1QJ, United Kingdom

We have characterized the effects of the antimitotic drug paclitaxel (Taxol™) on the Ca2+ signaling cascade of terminally differentiated mouse pancreatic acinar cells. Using single cell fluorescence techniques and whole-cell patch clamping to record cytosolic Ca2+ and plasma membrane Ca2+-dependent Cl- currents, we find that paclitaxel abolishes cytosolic Ca2+ oscillations and in more than half of the cells it also induces a rapid, transient cytosolic Ca2+ response. This response is not affected by removal of extracellular Ca2+ indicating that paclitaxel releases Ca2+ from an intracellular Ca2+ store. Using saponin-permeabilized cells, we show that paclitaxel does not affect Ca2+ release from an inositol trisphosphate-sensitive store. Furthermore, up to 15 min after paclitaxel application, there is no significant effect on either microtubule organization or on endoplasmic reticulum organization. The data suggest a non-endoplasmic reticulum source for the intracellular Ca2+ response. Using the mitochondrial fluorescent dyes, JC-1 and Rhod-2, we show that paclitaxel evoked a rapid decline in the mitochondrial membrane potential and a loss of mitochondrial Ca2+. Cyclosporin A, a blocker of the mitochondrial permeability transition pore, blocked both the paclitaxel-induced loss of mitochondrial Ca2+ and the effect on Ca2+ spikes. We conclude that paclitaxel exerts rapid effects on the cytosolic Ca2+ signal via the opening of the mitochondrial permeability transition pore. This work indicates that some of the more rapidly developing side effects of chemotherapy might be due to an action of antimitotic drugs on mitochondrial function and an interference with the Ca2+ signal cascade.

Antimitotic drugs are used extensively for the treatment of cancer. For example, paclitaxel (Taxol) is used in the treatment of breast and ovarian cancers and for AIDS-related Kaposi’s sarcoma, and vinblastine is used in the treatment of Hodgkin’s disease (1). The mechanism of action of antimitotic drugs, that leads to cancer cell death, is not clear. It is known that paclitaxel stabilizes microtubule dynamics thereby preventing the proper formation of the mitotic spindle apparatus and arresting cancer cells at the G2-M phase of the cell cycle (2, 3). While it is thought that this action of paclitaxel on the cell cycle machinery precedes an apoptotic response of cells (4, 5), some recent work has suggested that paclitaxel-induced apoptosis results from more direct effects of the drug on the mitochondria. In this context paclitaxel has been shown to bind to Bcl-2 (6) and this binding may regulate Bcl-2 effects on the mitochondrial permeability transition pore (PTP) (7, 8). Furthermore, deletion of the loop region of Bcl-2 (which prevents Bcl-2 phosphorylation) blocks the apoptotic action of paclitaxel on cancer cells (9). Other proteins may also be involved in the paclitaxel effects on mitochondria, such as APAF-1 (10). In isolated mitochondria paclitaxel acts to release cytochrome c (11). This effect is blocked by cyclosporin A providing further evidence that paclitaxel directly targets mitochondria, independent of actions on microtubules.

The effect of antimitotic drugs on microtubule dynamics would confer drug specificity on actively dividing cancer cells. However, the actions of these drugs on Bcl-2 and on the mitochondria might be expected to be non-selective and affect all cells. Indeed paclitaxel treatment is associated with serious side effects, including neuropathy (12) and low white blood cell counts (13). These side effects occur rapidly, appear to be due to drug action on terminally differentiated cells, and are slowly reversible. Thus it is unlikely that these side effects are mediated by either mitotic block or apoptosis. Given that the clinical use of antimitotic drugs is limited by these side effects, understanding the mechanisms by which these drugs act is an important step toward optimizing the therapeutic benefits.

In our studies, on terminally differentiated epithelial cells, we now show rapid actions of paclitaxel on the cytosolic Ca2+ signal that can be accounted for by effects of paclitaxel on the PTP of the mitochondria. Given the universality of Ca2+ signaling, it is likely that this action of paclitaxel accounts for some of the side effects of antimitotic drugs.

**EXPERIMENTAL PROCEDURES**

**Cell Preparation—**Male outbred albino mice (25 g) were killed by cervical dislocation and the pancreas dissected. Mouse pancreatic acinar cells were prepared by collagenase (Worthington, CLSPA, Lorne Labs.) digestion at 33 °C for 7 min as previously described (14). Cells were plated onto poly-L-ornithine (Sigma, United Kingdom)-coated dishes and were used within 3 h of isolation from the animal.

Pipes, 1,4-piperazinediethanesulfonic acid; Ins(1,4,5)P3, inositol 1,4,5-trisphosphate; Ins(2,4,5)P3, inositol 2,4,5-trisphosphate.
Patch Clamp—Standard whole cell patch clamp techniques (15) were employed and all experiments were carried out at room temperature (∼21 °C). Pipettes had a resistance of 3–6 MΩ (pipette puller, Brown and Flaming). After breaking through to the whole cell configuration bonyl cyanide (Flaming). After breaking through to the whole cell configuration, the intracellular stores loaded to steady-state with 45Ca²⁺.

The extracellular solution contained (mm) NaCl 135, KCl 5, MgCl₂ 1, CaCl₂ 1, glucose 10, NaOH-HEPES 10, pH 7.4. In the Ca²⁺-free solution, used in the experiments of Fig. 2, no Ca²⁺ was added. Cells were held at a membrane potential of −30 mV and currents were sampled by an A/D converter (EPC-9, HEKA) at 2 KHz. In most experiments Ins(2,4,5)P₃ (gift from Professor R. Irvine, Cambridge, UK) was added to the pipette solution (10 µM) to establish trains of Ca²⁺ spikes. These spikes have previously been shown to result from InsP₃-dependent Ca²⁺ release in the secretory pole (16).

Fluorescence Imaging—Ca²⁺ imaging experiments were performed as previously described (17). Briefly, inclusion of 40–50 µM Calcium Green in the pipette solution allowed imaging of the cytosolic Ca²⁺ signal. Cells were illuminated at 488 nm (Coherent Innova 70) and imaged through a Nikon ×40 UV, 1.3NA, oil immersion objective through a 510-nm long pass filter. Full frame images (128 × 128 pixels) were captured on a cooled CCD camera (70% quantum efficiency, 5 electrons readout noise; MIT, Lincoln Laboratories) with a pixel size of 200 × 200 Å. The images were sampled at rates of 1 to 500 Hz. After recording to computer, the data were analyzed with custom software with the appropriate smoothing. Images were displayed as ∆F/F₀ images (100 × (F – F₀)/F₀), where F is the recorded fluorescence and F₀ was obtained from the mean of 20 sequential frames where no activity was apparent.

Single-cell Fluorescence Measurement—Cells were loaded with actetoxymethyl esters of various fluorescent probes (Molecular Probes) and the fluorescent signal measured from single cells with a Cairn Dual Emission Fluorescence System (Cairn Research, Faversham, UK). Measurement of mitochondrial membrane potential (ΔΨm) was carried out using the dye JC-1 loaded at a concentration of 1 µM for 20 min at room temperature (22 °C). The dye was excited at a wavelength of 488 nm and the emitted light collected at 590 and 530 nm. The ratio of red (590 nm) to green (530 nm) gives an index of the mitochondrial membrane potential: the higher the mitochondrial potential the greater proportion of JC-1 aggregates in the mitochondria, the greater the intensity of the red light signal and so with active mitochondria we see a relatively high ratio of 590/530 nm (18). To measure mitochondrial Ca²⁺ signals we used Rhod-2AM at a concentration of 10 µM loaded at 37 °C for 40 min. This protocol specifically loaded the dye into the mitochondria by the overlapping fluorescent signal from the mitochondrial dye Mitotracker (Fig. 6). In these experiments data are presented as P/F₀, where P is the recorded fluorescence and F₀ was measured before drug application.

Estimates of Intracellular Paclitaxel Concentrations—In our experiments we used 10–20 µM paclitaxel applied to the bathing solution of the cell, and the intracellular concentation reached over a period of 10 min we used Oregon Green-conjugated paclitaxel (Molecular Probes) and imaged intracellular fluoresencing using a Zeiss LSM 510 confocal microscope. The intracellular fluorescence was measured over time within an 8-µm spot centered on the cell with an optical section of 1 µm. A calibration curve was made using a range of Oregon Green paclitaxel concentrations and recording the fluorescence signal as above. For two independent experiments we obtained a value of 322.5 nM as the peak intracellular concentration reached over 10 min. This value represents an upper limit for the cytosolic concentration of the drug, as it would be expected to bind to microtubules.

Ca²⁺ Flux Experiments—Hepatocytes were isolated from livers of male Wistar rats and then permeabilized by incubation with saponin (10 µg/ml) in cytosol-like medium containing 140 mM KCl, 20 mM NaCl, 2 mM MgCl₂, 1 mM EGTA, 20 mM Pipes (pH 7 at 37 °C). The cells were washed and resuspended (10 million cells/ml) in cytosol-like medium supplemented with CaCl₂ (free [Ca²⁺] ~ 200 nM), ATP (7.5 mM), carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP, 10 µM), and 45Ca²⁺ (7.5 cCi/ml) (19). FCCP was used in these experiments to exclude Ca²⁺ flux from mitochondria. After 5 min at 37 °C, during which time the intracellular stores were loaded to steady-state with Ca²⁺, the solution of 2.5 µM was added to inhibit further Ca²⁺ uptake together with paclitaxel (20 µM) or its solvent (Me₂SO) and either a maximal (5 µM) or submaximal (100 nM) concentration of Ins(1,4,5)P₃. After a further 5 min, the incubations were terminated by rapid filtration and the 45Ca²⁺ contents of the stores determined. After correction for the 45Ca²⁺ non-specifically bound to the cells (determined by addition of 10 µM iodo-

mycin), the 45Ca²⁺ contents of the stores were expressed as percentages of the control 45Ca²⁺ content.

Immunocytochemistry—Cells were washed quickly in PBS (including calcium and magnesium) and then in K-Pipes buffer (K-Pipes 80 mM, EGTA 5 mM, MgCl₂ 2 mM, pH 6.5). Cells were then fixed in 4% paraformaldehyde in K-Pipes buffer for 30 min and permeabilized in 0.1% Triton X-100 in PBS for 5 min. After a 1-h block in 2% donkey serum plus 2% fish skin gelatin in PBS, cells were incubated in primary antibody (β-tubulin mouse monoclonal, clone TUB 2, Sigma, UK) for 1 h. After washing, a secondary antibody (donkey anti-mouse Fab fragment, Jackson ImmunoResearch, West Grove, PA) conjugated to a fluorescent-alkyloxyaniline for 45 min. The images of Fig. 6 were obtained with a Zeiss LSM510 confocal microscope with a ×63 planapochromat oil immersion lens and excitation light provided by an argon and a HeNe laser. The emitted light collected at the indicated wavelengths. Confocal sections (1 µm in depth) were obtained in the mid-section of the cells and image overlays were produced with proprietary Zeiss software.

RESULTS

We have previously used the whole cell patch clamp method to record the activation of Ca²⁺-dependent chloride currents (Cl(Ca)) in single mouse pancreatic acinar cells (16). The Cl(Ca) current is commonly used as an indirect measure of cytosolic Ca²⁺ and is a particularly good index of the small subplasmalemmal Ca²⁺ signals that occur in the apical pole of polarized epithelia (17). Fig. 1 shows the typical effects of paclitaxel on the whole cell Cl(Ca) currents in a single pancreatic acinar cell. In a cell stimulated with Ins(2,4,5)P₃ to produce trains of Ca²⁺ spikes (16), paclitaxel induced a rapid increase in the Cl(Ca) current followed by an abolition of the spike response (n = 14/26, Fig. 1A). In the other 12 cells paclitaxel led to a gradual loss of the Cl(Ca) current spikes (Fig. 1B). Applied alone paclitaxel had no effect (n = 3 cells, Fig. 1C) and Me₂SO, the solvent in which paclitaxel was dissolved, at 1% (v/v) (a 5–10 greater concentration than that used in the paclitaxel experiments) had no significant effect on the current spikes (Fig. 1D).

These data indicate that paclitaxel can, in many cells, promote a rapid activation of the Cl(Ca) current. Since the Cl(Ca) channels are primarily under the control of cytosolic Ca²⁺ (20) this suggests that paclitaxel elicits a Ca²⁺ signal. To test this directly we carried out Ca²⁺ imaging experiments using the Ca²⁺-sensitive fluorescent dye Calcium Green introduced into the cell cytosol via infusion through the solution of the patch pipette (21). We further probed the possible source of the Ca²⁺ signal induced by paclitaxel by removing extracellular Ca²⁺ in these experiments. Fig. 2 shows a typical response, the infusion of Ins(2,4,5)P₃ resulting in the induction of small, short-lasting Ca²⁺ spikes in the apical region of the cell (indicated secretory pole, SP, in Fig. 2). Application of 10 µM paclitaxel induced a rapid Ca²⁺ response that spread from the apical region to all regions of the cell (n = 5/6 cells). In these experiments we were limited to capturing images over an 80-s period. However, a sequence of images captured later in the experiment, showed no evidence of a Ca²⁺ signal, indicating that the paclitaxel-evoked Ca²⁺ rise was transient and the local Ca²⁺ spikes were abolished (data not shown). This is entirely consistent with the data shown in Fig. 1A. Since there is no intracellular Ca²⁺ we conclude that the paclitaxel induced Ca²⁺ response must originate from an intracellular Ca²⁺ store.

The major Ca²⁺ store in pancreatic acinar cells is in the endoplasmic reticulum (ER) where Ca²⁺ is released in an IP₃-dependent manner (22, 23). To test if paclitaxel might be acting directly on IP₃-dependent stores we utilized the permeabilized hepatocyte preparation where the Ca²⁺ stores were preloaded with 45Ca²⁺ and the loss of radiolabeled Ca²⁺ in response to Ins(1,4,5)P₃ was measured (19). The data in Table I show that a saturating concentration of 5 µM Ins(1,4,5)P₃ releases about 30% of the Ca²⁺ from the store and that this response is...
unaffected by the presence of paclitaxel. At an intermediate concentration of Ins(1,4,5)P₃ (100 nM) about 20% of the store Ca²⁺ content is released and again paclitaxel has no significant effect on this. We conclude that the intracellular Ca²⁺ store affected by paclitaxel is not likely to be IP₃-dependent. However, our data do not exclude the possibility that, while paclitaxel may not act directly on IP₃-dependent release of Ca²⁺, it may act indirectly by physically changing the store morphology and this in turn may affect store behavior. In this context we have previously shown that microtubule depolymerizing agents have dramatic effects on Ca²⁺ signaling by locally and rapidly reorganizing the ER (24). We therefore went on to study the possible effects of paclitaxel on microtubular and ER organization.

To measure any effects of paclitaxel on microtubule and ER organization we carried out experiments to immunolocalize

TABLE I

| IP₃-dependent Ca²⁺ release is unaffected by paclitaxel |
|-------------------------------------------------------|
| ²⁴Ca²⁺ was preloaded into the Ca²⁺ stores of permeabilized hepatocytes. The figures show the ²⁴Ca²⁺ remaining after the various conditions, expressed as % of the total content. 5 μM Ins(1,4,5)P₃ is a saturating concentration and the entire Ins(1,4,5)P₃-dependent Ca²⁺ store is emptied (19). In our experiments this represents about 30% of the total loaded ²⁴Ca²⁺. With 100 nM Ins(1,4,5)P₃ about 20% of the total store is emptied. In all conditions paclitaxel had no significant effect on Ins(1,4,5)P₃-dependent release. The ²⁴Ca²⁺ remaining after application of 5 μM Ins(1,4,5)P₃ is presumed to represent Ca²⁺ uptake into Ins(1,4,5)P₃-independent pools such as secretory vesicles and endosomes. Data are expressed as the mean ± S.E. |
| Control | Paclitaxel |
|---------|-----------|
| 100 nM IP₃ | 107 ± 4 | 83 ± 3 | 70 ± 3 | 72 ± 4 |

Fig. 1. Paclitaxel rapidly activates the Clₐ current in whole cell patch clamp recordings of single isolated mouse pancreatic acinar cells. Cells were held under voltage clamp at a membrane potential of −30 mV. A, when Ins(2,4,5)P₃ (10 μM) was included in the patch pipette solution trains of spikes (downward deflections in the current, i.e. the outward movement of Cl⁻) were induced, and in the majority of cells the application of paclitaxel (10–20 μM) rapidly promoted a transient increase in current amplitude and broadened the spike width. B, in other cells paclitaxel induced a rapid abolition of spiking. C, applied alone to the bathing solution paclitaxel had no effect on the currents; and D, the Me₂SO carrier applied alone had no effect on spiking.

Fig. 2. Paclitaxel induces a rapid global Ca²⁺ response. In these experiments cells were filled with the Ca²⁺-sensitive dye calcium green and IP₃ (10 μM) and fluorescent images collected every 200 ms. The phase image (upper left) shows the orientation of the cell with the apical (secretory) pole (SP) on the left-hand side and the basal pole (BP) on the right. This cell was part of a 3-cell cluster and the other cells can be seen on the left. Image a was obtained during a quiescent period and shows low Ca²⁺ (pseudocolor blue) across the cell. The box in this image shows the area used in producing the average fluorescent signal versus time shown in the graph at the bottom of the figure. Images b, d, and f were obtained at the peak of the Ca²⁺ spikes and illustrate that the signal does not spread beyond the secretory pole region. In contrast, image h was obtained at the peak of the paclitaxel-induced response and shows a large Ca²⁺ rise (pseudocolor red) globally across the whole of the cell.
Paclitaxel Action on Cytosolic Calcium Signaling

Fig. 3. Paclitaxel does not dramatically change the microtubule distribution. In control cells (upper panel) a 3-cell cluster is shown with single confocal image planes taken at the bottom (~2 μm from the bottom of the coverslip), middle, and top (~2 μm down from the top of the cells). The images show a concentration of microtubules in the secretory pole (central granular region) which is where the microtubule organizing centers are thought to be. From the secretory pole microtubules radiate in an apparently disordered array. After 5 min (middle panel) and 15 min (lower panel) treatment with paclitaxel there is no dramatic effect on this microtubule organization although there is an apparent change in the morphology of microtubules in the basal pole.

β-tubulin and to image the fluorescent probe, ER tracker, respectively. Standard microtubule-preserving techniques were used to fix the acinar cells (K-Pipes buffer) and we probed with a monoclonal antibody against β-tubulin. We then imaged clusters of acinar cells taking confocal sections through the cluster of cells at various depths. For clarity, Fig. 3 shows confocal sections only through the lower, middle, and the upper parts of the cell clusters. In the phase images shown the apical domain is seen in the central part of the cell clusters and is delineated by the presence of the phase-dark secretory granules. In control cells an extensive network of microtubules was observed with predominance in the apical domain, the region presumed to contain the microtubule organizing centers (25) (Fig. 3). Application of paclitaxel (20 μM) did not dramatically alter this pattern of staining although the impression was that after 5 min treatment there was a structural reorganization that led to a criss-cross pattern of microtubules in the basal pole (two independent preparations). To test if this possible rearrangement of the microtubular system has any effect on the ER distribution we used ER tracker that has the key advantage of being widely distributed across the cell with exclusion from the nucleus and secretory granules. A, after paclitaxel treatment no change in the pattern of fluorescence was observed. B, the signal was quantified by measuring the average fluorescence in a 5-μm diameter region in the secretory pole (SP) and ratios this to a 5-μm diameter region in the basal pole. Individual points on the graph were obtained from 7 cells (control, circles) and 10 cells (paclitaxel, triangles) with a minimum of 3 cells for any data point. We conclude that paclitaxel does promote changes in the microtubular system but, at least over this time frame, there is no reorganization of the ER. These observations are consistent with early observations, in other cell types, of paclitaxel effects on microtubules and ER organization (27).

Another intracellular Ca\textsuperscript{2+} store, significant in many cell types, is the mitochondria. In pancreatic acinar cells mitochondria have been shown to contain low levels of Ca\textsuperscript{2+} at rest, but to rapidly take up Ca\textsuperscript{2+} during P\textsubscript{i}-evoked Ca\textsuperscript{2+} responses (28). If paclitaxel acted to release Ca\textsuperscript{2+} from mitochondria this would be consistent with the cytosolic Ca\textsuperscript{2+} responses we observed. We therefore set out to determine possible effects of paclitaxel on mitochondria. If paclitaxel were stimulating a Ca\textsuperscript{2+}-dependent efflux; (29). Ca\textsuperscript{2+} efflux through any of these pathways would be expected to affect the mitochondrial membrane potential (Δψ\textsubscript{m}). To record this we utilized the potential-sensitive probe JC-1. We observed a rapid decrease in the ratio of emitted light collected at 590 and 530 nm on addition of the mitochondrial uncoupler FCCP (1 μM, Fig. 5A) that represents a drop in Δψ\textsubscript{m}. In separate experiments, 10 μM paclitaxel (n = 8/11 cells, Fig. 5B) induced a decrease in Δψ\textsubscript{m}.
nM acetylcholine induced an increase in the mitochondrial Ca\textsuperscript{2+} of the cells with the agonist acetylcholine. In 3/4 cells, 100 cells is predominantly from the mitochondria. The result indicates that the Rhod-2 signal we record from single cells co-localizes with the Mitofluor fluorescence (overlay) indicating the our dye loading conditions for Rhod-2AM selectively loads the mitochondria. The Rhod-2 signal can therefore be used as a measure of mitochondrial Ca\textsuperscript{2+}.

To determine if these effects of paclitaxel on the \( \Delta \psi_m \) might be associated with a loss of mitochondrial Ca\textsuperscript{2+}, we carried out experiments to directly measure mitochondrial responses. We used Rhod-2/AM, a Ca\textsuperscript{2+}-sensitive dye that can be specifically loaded into the mitochondria. To verify that our dye loading protocol led to a specific loading of the mitochondria we carried out control experiments where we also loaded the cells with Mitofluor Green/AM, a mitochondrial specific dye (30). The excitation/emission wavelengths of these dyes are widely separated and we ensured, using experiments with only a single dye, that there was no “bleed” from one fluorescence channel to the other. We then took confocal images of Rhod-2 and Mitofluor Green fluorescence in the same cell. The results indicated that Rhod-2 and Mitofluor Green were loaded into the same cellular compartment with a characteristic distribution (Fig. 6) similar to that previously described for mitochondria in these cells (31). This result indicates that the Rhod-2 signal we record from single cells is predominantly from the mitochondria.

First we measured the Rhod-2 response following stimulation of the cells with the agonist acetylcholine. In 3/4 cells, 100 nM acetylcholine induced an increase in the mitochondrial Ca\textsuperscript{2+} as measured by an increase in the Rhod-2 fluorescence (Fig. 7A). Subsequent addition of 10 \( \mu \)M paclitaxel induced a drop in mitochondrial Ca\textsuperscript{2+} (\( n = 4/4 \) cells) and a further decline was seen with the addition of 1 \( \mu \)M FCCP (\( n = 3/3 \) cells). In cells not stimulated with agonist the measured Rhod-2 response from a single cell showed a rapid decline in response to paclitaxel (\( n = 5/8 \) cells) and a further decline in response to 1 \( \mu \)M FCCP (\( n = 6/7 \) cells, Fig. 7B). From these results we conclude that paclitaxel does induce a loss of Ca\textsuperscript{2+} from the mitochondria. To test for the possible route of exit of Ca\textsuperscript{2+} we pretreated the cells with cyclosporin A (10 \( \mu \)M), a drug that binds to cyclophilin D and leads to the block of the mitochondrial PTP. The subsequent addition of paclitaxel either failed to induce any decrease in the Ca\textsuperscript{2+} signal (\( n = 5/8 \)) or slowed the rate of decrease of the Ca\textsuperscript{2+} signal (\( n = 3/8 \), Fig. 7C). To quantify these effects we measured the paclitaxel-induced decrease in Rhod-2 fluorescence 30 s after drug application in the absence and presence of cyclosporin A (Fig. 7D). An FCCP induced decrease in Ca\textsuperscript{2+} was still observed after pretreatment with cyclosporin A (\( n = 6/6 \)), presumably because Ca\textsuperscript{2+} can still exit via other efflux mechanisms.

If the paclitaxel action on the cytosolic Ca\textsuperscript{2+} signal was mediated by an effect on mitochondria then cyclosporin A might be expected to protect the cell from paclitaxel action. We tested this first with 10 \( \mu \)M cyclosporin applied during a train of spikes induced by Ins(2,4,5)P\textsubscript{3}. However, the drug had effects alone and acted to increase spike activity (\( n = 3 \), data not shown). A lower concentration of cyclosporin A (5 \( \mu \)M) was therefore used. This had no effect on spike activity. After treating the cells for 5 min in cyclosporin A, we then applied paclitaxel. In contrast to control cells paclitaxel did not abolish spiking and had only a small transient effect on the signal (\( n = 3/4 \) cells, Fig. 8). This data supports our hypothesis that the primary action of paclitaxel on the Ca\textsuperscript{2+} signal cascade is mediated via an effect on the PTP.

**DISCUSSION**

The major finding of this study is that the anticaner drug paclitaxel rapidly releases Ca\textsuperscript{2+} from the mitochondria of pancreatic acinar cells. Although the mechanism underlying this effect is not completely clear, the ability of cyclosporin A to block the Ca\textsuperscript{2+} release points to an effect of paclitaxel on the PTP. We suggest that paclitaxel acts by partially opening the PTP. Ion flow through the PTP then causes a drop in mitochondrial membrane potential and a release of mitochondrial Ca\textsuperscript{2+}. 

**FIG. 6. Rhod-2 fluorescence co-localizes with Mitofluor fluorescence.** Imaging of Rhod-2 (Ca\textsuperscript{2+}-sensitive dye) and Mitofluor (a mitochondrial specific fluorescent probe) in a cluster of living acinar cells shows the characteristic distribution of mitochondria around the secretory pole. In a mid-confocal section through the cells, Rhod-2 clearly co-localizes with the Mitofluor fluorescence (overlay) indicating the our dye loading conditions for Rhod-2AM selectively loads the mitochondria. The Rhod-2 signal can therefore be used as a measure of mitochondrial Ca\textsuperscript{2+}.
Paclitaxel action on cytosolic calcium signaling has been noted before. Paclitaxel phosphorylates Bcl-2 in cancer cells and induces apoptosis, possibly by acting on the PTP (4–6). Andre et al. (11) have shown that paclitaxel induces cytochrome c release from a preparation of isolated mitochondria. The same study also showed that this cytochrome c release was blocked by pretreatment with cyclosporin A. These direct or indirect actions on mitochondria (32, 33) may in turn generate the cytosolic Ca\(^{2+}\) responses we observe.

Our measurements of the effects of paclitaxel on the Ins(2,4,5)P\(_3\)-induced spikes indicate that in more than 50% of cells the drug induces a release of Ca\(^{2+}\) from an intracellular store followed by abolition of the spikes. In the remaining cells paclitaxel led to a rapid abolition of spike activity. Why does the drug have these different actions? The same action on mitochondria may underlie both effects, with the difference being heterogeneity of mitochondrial Ca\(^{2+}\) loading. In cells where the mitochondria were releasable with Ca\(^{2+}\), paclitaxel-induced opening of the PTP would elicit a cytosolic Ca\(^{2+}\) signal. However, in cells where the mitochondria were less well loaded the expected cytosolic Ca\(^{2+}\) signal may be too small to detect. Pharmacological block of mitochondrial function has been shown to enhance cytosolic Ca\(^{2+}\) responses in astrocytes (30) and acinar cells (31). These effects may be equivalent to the transient cytosolic Ca\(^{2+}\) response to paclitaxel we see in the acinar cells. In all of our experiments we observed an eventual abolition of the Ca\(^{2+}\) spikes. This appears to conflict with the enhancement of responses described above. However, block of mitochondrial function has been shown to inhibit Ca\(^{2+}\) uptake into Ca\(^{2+}\) stores (34) and since the Ca\(^{2+}\) spikes in acinar cells are dependent on cycles of Ca\(^{2+}\) release and Ca\(^{2+}\) uptake this effect would lead to a loss of spiking. Mitochondria are physically held in close proximity to sites of Ca\(^{2+}\) release (35) and therefore are in a good position to modulate both Ca\(^{2+}\) release and Ca\(^{2+}\) reuptake (36).

The actual concentration of intramitochondrial Ca\(^{2+}\) results from a balance of Ca\(^{2+}\) uptake by the uniporter and a variety of Ca\(^{2+}\) efflux mechanisms (29). During periods of cell stimulation where the cytosolic Ca\(^{2+}\) is high, the mitochondrial Ca\(^{2+}\) rises (35). After stimulation, the intramitochondrial Ca\(^{2+}\) returns back to lower levels. It is not clear if the PTP contributes to the endogenous mechanisms of Ca\(^{2+}\) efflux from mitochondria but it may be important in processes of charge balance that would be required for Ca\(^{2+}\) exit. What is clear is that a drug-induced opening of the PTP, as we postulate for the action of paclitaxel, would both reduce the mitochondrial potential and lead to Ca\(^{2+}\) efflux. This would modify the cytosolic Ca\(^{2+}\) responses, as we show in this paper. In addition, since the intramitochondrial Ca\(^{2+}\) signal has been shown to actively regulate mitochondrial functions such as Ca\(^{2+}\)-dependent dehydrogenases (37), these would be expected to be affected by paclitaxel, and would lead to a loss of ATP production and the subsequent compromise of many other cell functions.

Our experiments indicate that the PTP plays a role in the paclitaxel-mediated responses but exactly how the PTP is affected is unclear. One possibility, put forward by Andre et al. (11) to explain paclitaxel effects on isolated mitochondria, was that the mitochondria might contain tubulin within their structure. In this model the binding of paclitaxel to this tubulin might influence PTP function either through a tubulin interaction with the voltage-dependent anion channel present on the outer mitochondrial membrane (7) or through the adenine nucleotide transporter at the inner membrane, both of which are thought to form the PTP. While this is an attractive hypothesis, other experiments clearly indicate Bcl-2 as a possible target for paclitaxel (6, 9). Bcl-2 is resident in the mitochondrial membrane and it is thought that drug-induced phosphorylation may lead to PTP

---

**Fig. 7.** Mitochondrial Ca\(^{2+}\) is decreased by paclitaxel and by FCCP. In these experiments cells were loaded with Rhod-2 under exactly the same conditions as in experiments of Fig. 6. A, single cell fluorescence measurements showed an increase in mitochondrial Ca\(^{2+}\) on addition of 100 nM acetylcholine and a drop in Ca\(^{2+}\) on addition of paclitaxel to the bathing solution and a further drop when FCCP was added. B, in unstimulated cells the same paclitaxel and FCCP induced drop in Ca\(^{2+}\) was observed. C, pretreatment with cyclosporin A inhibited the paclitaxel effect but did not block FCCP-induced reductions in Ca\(^{2+}\). D, graph showing the mean (± S.E., n = 8) drop in Rhod-2 fluorescence induced by paclitaxel alone, or in the presence of cyclosporin A. The difference in the two points was significant at p < 0.05 (Student’s t test).

**Fig. 8.** Pretreatment with cyclosporin A (5 μM) prevents the paclitaxel-induced effects on Ca\(^{2+}\) spiking. Cl\(-\) currents spikes induced with Ins(2,4,5)P\(_3\), current spikes induced with Ins(2,4,5)P\(_3\), were recorded in whole-cell patch clamp recordings of single isolated mouse pancreatic acinar cells and cyclosporin A applied to the bathing solution. Subsequent application of paclitaxel did not abolish spiking (n = 3/4). These effects may underlie some of the side effects of anti-mitotic drug treatments.
show that paclitaxel induces a rapid and dramatic change in \( \Delta \Psi_m \) seen (39, 40). In one study the change in \( \Delta \Psi_m \) was associated with mitochondrial swelling and was specifically induced by high, but not low, concentrations of Bax, the proapoptotic agent (39). In another study different states were differentially induced by A23187 or arachidonic acid and cell death was only associated with the changes in \( \Delta \Psi_m \) (40). We show that paclitaxel induces a rapid and dramatic change in \( \Delta \Psi_m \), Andre et al. (11) showed paclitaxel-induced mitochondrial swelling, and combined, these data suggest paclitaxel acts to induce a more sustained opening of the PTP. This would lead to paclitaxel-induced apoptosis as shown in cancer cells (10). How-
Paclitaxel Affects Cytosolic Calcium Signals by Opening the Mitochondrial Permeability Transition Pore
Jackie F. Kidd, Mary F. Pilkington, Michael J. Schell, Kevin E. Fogarty, Jeremy N. Skepper, Colin W. Taylor and Peter Thorn

*J. Biol. Chem.* 2002, 277:6504-6510.
doi: 10.1074/jbc.M106802200 originally published online November 27, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M106802200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 42 references, 21 of which can be accessed free at http://www.jbc.org/content/277/8/6504.full.html#ref-list-1