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CYTOTOXIC ACTION OF PALYTOXIN IN CULTURED AORTIC SMOOTH MUSCLE CELLS

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ABSTRACT

Palytoxin (PaTX) produced by coelenterate species (genus *Palythoa*) is a potent marine toxin which induces intense vasoconstriction and hemorrhage which contribute to lethality. The A7r5 clonal cell line, derived from fetal rat aorta, was used to study the mechanism of PaTX toxicity. A7r5 cells exposed to nM concentrations of PaTX for 15 min at room temperature followed by wash and incubation in culture medium at 37°C (≥30 min) developed swelling of endoplasmic reticulum, clumping of cytoplasm, blebbing and shrinking of heterochromatin. These cells were nonviable as confirmed by inclusion of trypan blue and release of LDH. The ED50 for PaTX cytotoxicity was 7.1 nM using the vital dye exclusion and 4.1 nM for LDH release. Whole-cell patch clamp recordings from single A7r5 cells showed that PaTX (3-10 nM, 25°C, 15 min) depolarized cells and increased membrane conductance to Na^+ and K^+ >5-fold. Neither the PaTX-induced cytotoxicity nor the increased ionic permeability could be reversed by washing. However, prior incubation of cells with ouabain (10^{-4}M) or replacement of extracellular Na^+ with choline$^+$ or K^+ after exposure to PaTX did reduce the cytoxicity. Removal of extracellular Ca^{2+} reduced PaTX-induced cytotoxicity, but treatment with 10 to 50 μM verapamil did not, indicating that voltage-gated calcium channels were not involved. The mechanism of PaTX cytotoxicity in A7r5 cells seems to involve intracellular Na^+ overload and can be duplicated by other Na^+ ionophores such as nystatin. In light of the involvement of smooth muscle in PaTX poisoning, A7r5 cells could serve as a useful model to test specific drugs for protection against PaTX acute and delayed toxicity.

INTRODUCTION

Palytoxin (PaTX) is a water soluble toxin isolated from certain coelenterate species of the genus *Palythoa* and also found in a variety of other, unrelated marine animals (4). The toxin causes a variety of symptoms in mammals, varying with the species and route of administration (10). Of the symptoms that have been described, two have been most often cited as being associated with lethal effects of the PaTX. Vasoconstriction is usually seen within minutes of intravenous administration and results in an acute toxicity. In animals where the vasoconstriction is ameliorated with vasodilators, animals suffer extensive hemorrhage resulting in delayed toxicity that can occur days after exposure to PaTX (10).

At the cellular level, PaTX is known to increase membrane permeability to Na^+ in both excitable and inexcitable cells (3,4,8,9). The increased permeability is due to the appearance of a selective cation conductance which leads to a steady-state depolarization. It has been suggested that PaTX converts the Na^+-K^+ ATPase ion pump into an ionophore. The depolarization induced by PaTX is the most likely cause of smooth muscle contractions *in vitro* and vasoconstriction *in vivo*. However, in addition to direct effects on plasma membrane permeability to Na^+ and K^+, PaTX causes disruption of cell structure in skeletal muscle and hemolysis in erythrocytes (3,4). In this series of studies, we are interested in identifying the links between the membrane conductances produced by PaTX and the cellular disruption that may be responsible for delayed hemorrhage and death.

For this study we have chosen to use the A7r5 clonal cell line as an *in vitro* model system. A7r5 cells are a clonal line derived from rat aorta. These cells show many of the cellular markers characteristic of vascular smooth muscle (5), and since many of the actions of PaTX involve vascular damage, the A7r5 cell line should prove to be a useful model for the effects of PaTX on vascular tissue. The use of a clonal cell preparation also facilitates patch clamp measurements of membrane phenomena as well as enables the routine examination of cell morphology, vital dye staining and lactate dehydrogenase (LDH) release for estimates of cell viability.

MATERIALS AND METHODS

Cell Culture. The A7r5 cell line was obtained from American Type Culture Collection, Bethesda, MD, USA. The cells were grown in Dulbecco's Modified Eagles Medium supplemented with 5% fetal bovine serum at 37°C in an atmosphere of 90% air-10% CO_2. Cells were allowed to grow to confluence, 7 to 10 days, before use. Most of the experiments were carried out between 8 and 15 days.

Cytotoxicity. Before exposure to PaTX the cells were washed twice with exposure solution consisting of (mM) 130 NaCl, 5.4 KCl, 0.8 $MgCl_2$, 1.8 $CaCl_2$, 20 HEPES and 15 d-glucose. The pH was adjusted to 7.4 with NaOH. The cells were exposed to various

concentrations of PaTX for 15 min at room temperature (23°C). Following exposure, cells were washed and replaced by 1 ml serum-free DMEM or modified incubation saline as indicated. After incubation at 37°C for 2 hr the cells were observed visually. In experiments with verapamil and ouabain the washed cells were exposed to the drugs for 30 min prior to PaTX treatment. The drugs were present during PaTX exposure and during the 2-hour incubation period. In most experiments the viability of cells was assessed by measurement of LDH released into the supernatant. Assays of released LDH were preformed according to the procedure described in (1). For some experiments after removal of 0.2 ml of supernatant for LDH determination the cells were stained with trypan blue or nigrosin for dye-exclusion assays of relative cell viability. Replicates of at least 3 dishes were exposed to each experimental condition. LDH values were expressed as percent of maximal LDH released in a particular experiment and reflect the average response ± S.E.

Drugs and chemicals. Palytoxin and monensin were obtained from Calbiochem. A stock solution (100 μM) of PaTX was made in water and frozen at -20°C. Tissue culture media, fetal bovine serum, ouabain, nystatin and verapamil were from Sigma Chemical Co., St. Louis, MO.

Patch clamp recording. Coverslips, containing A7r5 cells, were transferred to a plexiglass recording chamber. PaTX and modified bathing solutions were applied at room temperature (24-26°C). Patch-clamp recording of whole-cell currents followed standard techniques (2,8).

RESULTS

A7r5 cells exposed to PaTX developed a series of morphological changes that correlated with cell death as assayed by dye exclusion and LDH release. Initial changes involved swelling of the endoplasmic reticulum, swelling of the nuclear envelope, contraction of the heterochromatin and, in many cases, detachment of the cell from the substrate. Essentially all cultures of A7r5 cells that showed these morphological changes also demonstrated marked cell death as indicated by lack of exclusion to vital dyes such as trypan blue and nigrosin. The loss of cellular LDH into the culture medium also correlated well with the morphological damage.

The concentration of toxin present during the exposure phase, the duration of the exposure, and the temperature of the incubation phase all affected the amount of cell damage caused by PaTX. Figure 1 shows the relative release of LDH as a function of PaTx concentration. In these experiments a fixed 15-min exposure at room temperature was followed by a 2-hour incubation at the indicated temperature. For incubations at the standard temperature of 37°C, the amount of LDH released was concentration dependent, reaching a half-maximal release at 4.1 nM. The curve had a more than 1:1 dependence on PaTX concentration and showed a Hill coefficient of approximately 1.43 in this experiment. The amount of PaTX that induced half-maximal release of LDH and the concentration

causing 50% of the maximal cell death assayed by single-blind cell counts in vital stain were 4.1 nM and 7.5 nM respectively. The LDH assays were considered more reliable because the vital stain underestimated the cell damage by not counting dead cells that detached from the substrate.

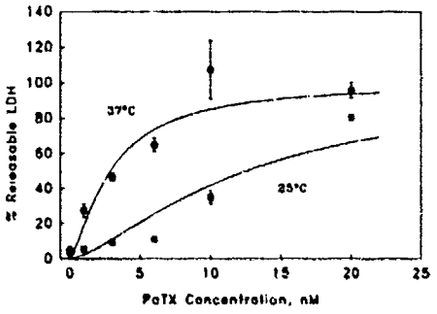

Figure 1. PaTX-induced cytotoxicity in A7r5 cells. The ED50's were 4.1 nM at 37°C and 44 nM at 25°C. LDH release was normalized to the releasable pool.

The amount of damage induced by a given concentration of PaTX increased at elevated temperatures. Figure 1 shows the difference in the concentration dependence of A7r5 cytotoxicity when the temperature was reduced from the normal 37°C incubation temperature to 25°C. The ED50 concentration of PaTX increased from 4.1 nM to 44 nM. Conversely, the maximum amount of LDH release at high concentrations of PaTX remained relatively constant with respect to temperature. It should be noted that these temperature effects were in the incubation phase after removal of free PaTX, and thus probably represent a temperature dependence in the cellular response to a fixed PaTX challenge during the exposure phase. As the exposure period at 25°C was lengthened, the amount of cytotoxicity increased, reaching a maximum at about 60 min. Increasing the incubation time up to 24 hours did not increase cytotoxicity. The dependence of cytotoxicity upon exposure time suggests that the dose-response curve of Figure 1 represents a rate vs. dose relationship rather than an equilibrium response to PaTX.

Electrophysiological recordings of membrane potentials in A7r5 cells indicated that substantial changes in the resting potential and membrane conductance occurred within minutes of PaTX application. In the presence of PaTX, the resting membrane depolarized and the resting membrane conductance increased. It is unlikely that there was any change in the gradients for Na^+, K^+ or Cl^- concentrations within the patch-clamped cells due to diffusion of ions from the large solution reservoir represented by the filling solution in the patch pipette. However, even in the presence of maintained intracellular and extracellular ion concentrations, A7r5 cells depolarized to approximately 0 mV.

Replacement of extracellular Na^+ with a relatively impermeant cation such as $choline^+$ resulted in a reversal of the PaTX-induced depolarization. This effect was not due to a washout of the PaTX conductance. Subsequent return to a sodium-containing solution outside the cell caused a prompt return to the depolarized state. Substitution of extracellular Na^+ with a permeant cation such as K^+ had little effect on the membrane depolarization seen with PaTX. Using the shifts in the reversal potentials of PaTX-induced conductance under conditions of extracellular ionic substitution it was possible to calculate the relative permeability ratios of ions within the PaTX ion channel. The relative order of permeability

was $Na^+ > K^+ >$ choline$^+$ in the ratios of 1.0:0.47:0.42. There was essentially no permeability to Cl^-.

Substitutions in the ionic composition of the medium bathing A7r5 cells during exposure and incubation altered the cytotoxicity produced by PaTX as well as membrane conductances. Figure 2 shows the effect of removing extracellular calcium (without buffering) on LDH release from paired cultures exposed to a range of PaTX concentrations. When Ca^{2+} was absent in both the exposure and incubation phases, the cells were protected from PaTX cytotoxicity over a wide range of PaTX concentrations. In order to test if toxic levels of extracellular Ca^{2+} entered A7r5 cells through voltage dependent calcium channels subsequent to prolonged PaTX-induced depolarization, verapamil was used to block Ca^{2+} entry through the L-type calcium channels that are known to exist in the A7r5 cell line (2,5). Unlike the situation with Ca^{2+} removal, there was no decrease in cytotoxicity induced by PaTX with either 10 or 50μM verapamil treatments.

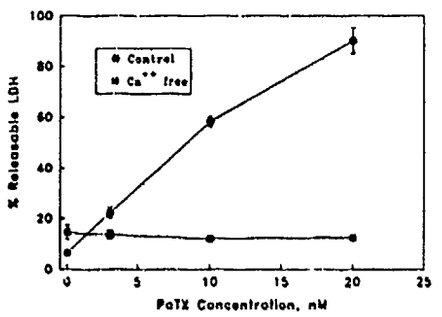

Figure 2. Effect of removing extracellular Ca^{2+} on PaTX-induced cytotoxicity. Ca^{2+} was removed during both exposure and incubation. Incubations were at 37°C.

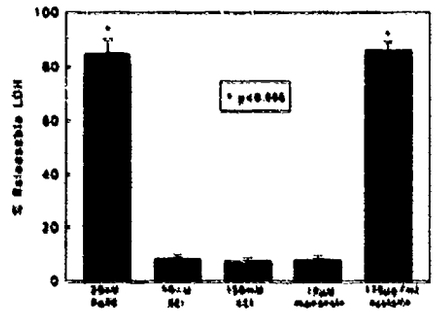

Figure 3. Comparison of cytotoxic effects of depolarizing agents with PaTX cytotoxicity in A7r5 cells. * indicates significant deviation from control LDH levels. All data incubated at 37°C.

Simple depolarization of A7r5 cells was not a cytotoxic stimulus. Figure 3 shows the results of several experiments where the cytotoxic effects of various depolarizing agents were compared with the effects of 20 nM PaTX. Both 50 and 150 mM KCl solutions applied to A7r5 cells resulted in essentially complete depolarization as determined by electrophysiological recordings. However, when cells were exposed and incubated in these solutions, there was no evidence of cytotoxic damage over controls. The $Na^+/H^+/K^+$ ionophore monensin at 10 μM caused depolarization but not a significant membrane conductance as measured on patch clamped cells. Monensin also produced no cytotoxic damage as assayed by LDH release. However, the application of the monovalent ionophore nystatin, which both depolarized the cells and induced a large membrane conductance to Na^+ and K^+, caused as much cytotoxicity as PaTX.

The presence of Na^+ in the extracellular solution during the incubation phase seems to be a critical factor in the development of cytotoxicity in the presence of PaTX. Figure 4 shows experiments where the effects of 20 nM PaTX present in the exposure phase could be overcome by ionic substitutions. Whenever Na^+ was substantially reduced and replaced with another permeant cation (K^+) or an impermeant cation (choline$^+$) the cytotoxic damage induced by PaTX was significantly reduced. This protectant effect was only seen when the substitution included the incubation phase of the experiment. Substitutions made during the exposure phase alone were generally without effect. Also shown in Figure 4 is the protective effect of preincubation with 100 μM ouabain. This treatment is known to block the contractions caused by PaTX in smooth muscle and, as shown here, is also effective in reducing the cytotoxic actions of PaTX.

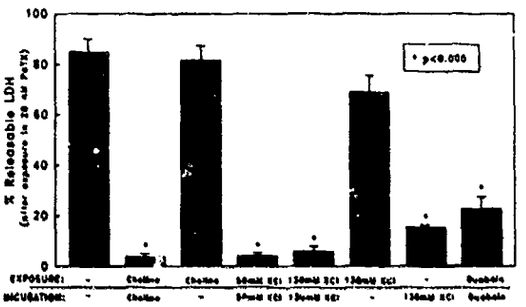

Figure 4. Cytoprotectant effects of ionic substitutions during exposure and/or during incubations after PaTX. Also shown is the effect of 100 μM ouabain. * indicates significant differences from PaTX alone.

DISCUSSION

The cytotoxic actions of PaTX seem to be related to its ability to increase the membrane permeability to monovalent cations. The initial insult to the cell membrane is rapid, as indicated by the electrophysiological measurements, and essentially irreversible. It seems highly significant that although the initial insult to the cell is irreversible, the cytotoxic damage is not inevitable. The incubation phase, where cellular damage was monitored, appears to be the time where cell death occurs. Cellular damage during incubation was temperature dependent, proceeding faster and at lower concentrations of PaTX when the temperature was elevated to 37°C. Electrophysiologically, the maximum membrane permeability had already occurred before the incubation phase (and at 25°C). Thus, the cytoxicity seems to follow the membrane permeability insult and seems to be a more temperature dependent phenomenon.

Cytotoxicity of PaTX in A7r5 cells is also dependent on the presence of two ions in the extracellular fluid, Ca^{2+} and Na^+. If either ion is missing, the cytotoxicity induced by PaTX is greatly reduced. In the case of the Ca^{2+} removal, the effect could be due to a reduction in the rate at which PaTX increases membrane permeability (3,6,8,9). However the complete lack of cytotoxicity in Ca^{2+}-free solutions is not completely consistent with this interpretation as the only action of Ca^{2+}. It seems more likely that Ca^{2+} is also involved

in the secondary effects of PaTX that lead to cytotoxicity. It is clear that the source of Ca^{2+} in the cytotoxicity is extracellular and that the Ca^{2+} is not entering through voltage gated channels, since verapamil has no effect on cytotoxicity and simple depolarization induced by other agents is not sufficient to replicate PaTX cytotoxicity.

The dependence of PaTX cytotoxicity on extracellular Na^+ is also indicative of the changes occurring during the toxic incubation after PaTX. Even when Na^+ is replaced with permeant monovalent cations, the cytotoxic actions of PaTX are blocked. This indicates a specificity for increased Na^+ permeability as an essential stage in PaTX-induced cell death. That this dependence is seen during the incubation phase suggests that cell-loading with Na^+ is an important step (and the initial conductance changes during exposure are only a precursor). The cytotoxicity seen with nystatin suggests that any mechanism that loads A7r5 cells by increasing sodium permeability will mimic the actions of PaTX. However, as seen with monensin, the permeability increase must be substantial to cause cell damage in the incubation phase.

One possible sequence of events that could explain all these observations is that initial exposure to PaTX causes the cell membrane to become permeable to Na^+. This permeability could be due to an alteration by PaTX of the membrane Na^+/K^+ ATPase, as has been proposed by other investigators (3), that converts the Na^+/K^+ ATPase into an ionophore. Our ouabain results are consistent with this interpretation of the primary action of PaTX. The prolonged influx of Na^+ during the incubation phase could then initiate a reversal of the Na^+/Ca^{2+} exchanger and load the cell with Ca^{2+}. The resulting high levels of intracellular Ca^{2+} would then cause the normal cascade of cell damage characteristic of calcium loading.

In summary, while our results are consistent with the idea that the primary insult to cells by PaTX is a large permeability increase to Na^+ and K^+, cytotoxicity is a secondary process that requires Na^+ flux into the cell and a coupled Ca^{2+} entry. It is also clear that calcium channel antagonists that are effective as antagonists of PaTX-induced vasoconstriction (6,7,10) are not effective in protecting vascular cells from cytotoxic damage subsequent to PaTX exposure.

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