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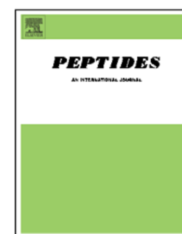

The antifungal protein AFP secreted by *Aspergillus giganteus* does not cause detrimental effects on certain mammalian cells

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ABSTRACT

The antifungal protein AFP is a small, cystein-rich protein secreted by the imperfect ascomycete *Aspergillus giganteus*. The protein efficiently inhibits the growth of filamentous fungi, including a variety of serious human and plant pathogens mainly of the genera *Aspergillus* and *Fusarium*, whereas AFP does not affect the growth of yeast and bacteria. This restricted susceptibility range makes it very attractive for medical or biotechnological use to combat fungal infection and contamination. We, therefore, analyzed whether AFP affects the growth or function of a number of mammalian cells. Here we show that the protein neither provokes any cytotoxic effects on human endothelial cells isolated from the umbilical vein nor activates the immune system. Moreover, potassium currents of neurons and astrocytes do not change in the presence of AFP and neither excitatory processes nor the intracellular calcium homeostasis of cultured skeletal muscle myotubes are affected by AFP. Our data, therefore, suggest that AFP is indeed a promising candidate for the therapeutic or biotechnological use as a potential antifungal agent.

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1. Introduction

For decades now we observe a steady increase in pathogenic microorganisms which do no longer respond to treatment with commonly used antimicrobial agents. The spectrum of affected sectors is substantial, where medical mycology is only one field in which we assert a rising number of rare and emerging pathogens, posing a serious challenge for ordinary antifungal agents [19]. In agriculture, huge economic losses are caused by the infestation of crop plants with fungal pathogens. The infection with plant pathogens such as *Fusarium* spp. not only causes severe yield losses and

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reduction in quality of grain, but far more dramatically, may also contaminate the grain with mycotoxins, which are hazardous to animal and human health [6]. Since the application of conventional antifungal agents is often ineffective and in some cases bears a high risk of toxicity for environment and consumer, new substances for the combat against pathogens are in great demand. These substances, ideally, combine a high degree of potency with a narrow susceptibility range and, at the same time, are thoroughly compatible with the environment.

Antifungal proteins produced in many organisms ranging from bacteria to higher organisms bear great potential for the prevention of fungal infections [24]. A prominent example for one such source is the ascomycete *Aspergillus giganteus*, an imperfect mold which secretes a small, basic protein, referred to as the 'antifungal protein' (AFP), first described in 1965 [18]. The most striking characteristic of AFP is its antifungal activity. The protein is highly efficient in inhibiting the growth of a variety of fungi, where predominantly the genera *Aspergillus* and *Fusarium* are affected by low concentrations of AFP. For example, growth of the plant pathogen *F. oxysporum* and of the human pathogen *A. fumigatus* is completely inhibited at an AFP concentration of 1 and 10 μg/ml, respectively [23,25]. Interestingly, the susceptibility range of AFP is strictly limited to filamentous fungi, thus the growth of yeast and bacteria is not affected [9,23,28]. AFP, therefore, seems to be an attractive alternative for chemical plant and food preservation and may also be a candidate for therapeutic use. The observation that AFP is indeed able to protect rice, geranium and tomato plants against *Magnaporthe grisea*, *Botrytis cinera* and *Fusarium oxysporum* infections, respectively [15,25,27], emphasizes its great biotechnological potential.

AFP consists of 51 amino acids (5.8 kDa) and is a highly basic peptide with an isoelectric point of 8.8 [17,28]. Four intramolecular disulfide bridges support its compact folding into a three-dimensional structure, forming a molecule, which exhibits a high resistance towards heat and protease treatment [2,25]. While the structure and the regulation of the respective *afp* gene have been studied in greater detail [12,13,14], less is known about the mode of action of AFP and its species-specificity. It has been shown recently that determinants for the species-specificity of AFP reside in the outer layer and the cell wall of sensitive fungi and that AFP causes permeabilization of the fungal plasma membrane [23,25]. In addition, it was reported that AFP is able to bind DNA under in vitro conditions and promotes charge neutralization and condensation of DNA [10], although, it remains unclear whether a DNA-binding affinity of AFP contributes to its antifungal activity.

Before a possible application of AFP, it is of particular importance to demonstrate that its growth inhibitory effect is, in fact, strictly limited to filamentous fungi. It is also essential to prove that neither growth nor function of mammalian cells is influenced by AFP and that the protein does not possess any immunogenic potential. In this study, we therefore applied AFP in a concentration range of 2–100 μg/ml (which is sufficient to severely inhibit the growth of AFP-sensitive fungi) on a wide variety of mammalian cells, specifically focusing on cytotoxicity, inflammatory effects and possible membrane effects. Our data show that the application of 2–100 μg/ml AFP on human endothelial cells does not provoke any cytotoxic effects. Moreover, it was also demonstrated that 2 μg/ml AFP does not cause an inflammatory response, as measured by the production of the pro-inflammatory cytokines IL-6 and IL-8 and that 10 μg/ml AFP does not affect calcium homeostasis in skeletal muscle fibres or potassium currents produced by neurones and astrocytes. These results suggest that the protein is a highly ~~a~~ promising candidate for future application in the combat against fungal pathogens.

2. Methods

2.1. Cultivation of A. giganteus and purification of AFP

A. giganteus strain 0902 was cultivated in 2 l Erlenmeyer flasks containing 500 ml medium (2% soluble starch, 1% beef extract, 2% peptone, 0.5% NaCl) for 96 h at 28 °C. The cultivation temperature was shifted to 37 °C, and after further incubation for 20 h the culture supernatant was harvested. The purification of AFP was achieved by using cationic exchange chromatography and gelfiltration according to the method described by Theis et al. [23].

2.2. Endothelial cell cytotoxicity assay

The cytotoxicity assay followed the protocol described earlier [1,22]. To this end, human umbilical vein endothelial cells (HUVECs) were removed from human umbilical vein by exposure to dispase II (Boehringer Mannheim, Vienna, Austria). Endothelial cells (identified as described in ref. [1] were cultured in medium 199 containing 15% fetal bovine serum (both from Life Technologies, Vienna, Austria), supplemented with L-glutamin, sodium-pyruvate, endothelial growth factor, a mixture of penicillin–streptomycin (100 U/ml each), and 5 U/ml heparin (Biochemie GmbH, Vienna, Austria) and grown in 24-well tissue-culture plates. Prior to exposure to AFP, cells were washed twice with Hank's balanced salt solution (HBSS, Life Technologies). Cells were treated with AFP at concentrations from 2 to 100 μg/ml in HBSS and in medium 199. After incubation for 7 and 16 h, respectively, the test solutions were replaced with 550 μl 3-[4,5-dimethylthiazol-2yl]-2,5-diphenyl-tetrazolium-bromide (MTT) in HBSS and cells were incubated for an additional 6 h [16]. The amount of reduced MTT was measured spectrophotometrically at 570 nm after the formazan was dissolved in 100 μl 10% sodium dodecyl-sulfate and 500 μl hot isopropanol containing 20 mM HCl. For positive control oxidized LDL in a concentration range of 50–500 μg/ml in HBSS was used.

2.3. Cytokine determination

Measurement of soluble IL-6 and IL-8 cytokine concentrations in the plasma samples was performed by ultra-sensitive double sandwich enzyme immunoassays (ELISA) according to the manufacturer's instructions (Biosource International Inc., Camarillo, CA) as reported earlier [22]. In brief, fresh blood samples of healthy donors were obtained from the Regional Blood Transfusion Centre and 3 ml of blood, prevented in its

clotting, were incubated with 300 μl of the test samples at 0.2–2 μg/ml final concentrations of AFP. Negative controls were incubated with 300 μl saline, whereas positive controls were treated with 300 μl lipopolysaccharide (LPS; *E. coli* 0128, Difco Laboratories #3131-25, Sparks, MI) solution to give a final concentration of 0.1 μg/ml. Samples were incubated for 1 h at room temperature on a shaker and the in vitro culture was continued for 24 h at 37 °C in a CO_2 incubator (5% CO_2). Plasma was collected by centrifugation at room temperature (1500 rpm, 23 min) and stored at −70 °C until cytokine determination. Cytokine concentrations were expressed as pg cytokine/ml detected in the undiluted individual plasma samples. The inflammatory effect of the test samples was characterized by the relative increase of cytokine concentration compared to the basal level detected in the untreated control plasma.

2.4. *Enzymatic isolation and tissue culturing of astrocytes*

Astrocyte cultures were prepared from the hippocampus of the rat as described earlier [22]. The hippocampus was dissected in an ice-cold medium (D1; in mM: NaCl, 136; KCl, 5.2; $Na_2HPO_4{\cdot}H_2O$, 0.64; KH_2PO_4, 0.22; glucose, 16.6; sucrose, 22; HEPES, 10; plus 0.06 U/ml penicillin and 0.06 μg/ml streptomycin), and then enzymatically dissociated in D1 solution, containing trypsin (0.025 g/ml; 30 min, 37 °C). The tissue pieces were then transferred to minimum essential medium (MEM) supplemented with 10% fetal calf serum (FCS) for 5 min (room temperature). Individual cells were separated by gentle agitation. The cell suspension was then diluted to 100,000 cells/ml, and 0.5 ml of this suspension was transferred onto cover-slips. Cells were allowed to grow for 4–5 days at 37 °C in a 5% CO_2 atmosphere whereby the feeding medium (MEM, supplemented with 10% FCS) was changed daily. ~70–80% confluent cultures were used for the electrophysiological experiments.

2.5. *Tissue culturing of skeletal muscle cells*

The procedure for obtaining satellite cells and the culturing of myotubes from these cells were the same as described in our earlier reports [4,21]. In brief, the muscle was dissociated at 37 °C using collagenase (0.75 mg/ml, Type II, Sigma, St. Louis, MO, USA) and trypsin (1 mg/ml, Difco, Detroit, MI, USA) in a calcium/magnesium free phosphate buffered saline (PBS). Dissociated cells were seeded onto sterile coverslips (32 mm diameter, 0.07 mm thickness; Biophysical Technologies Inc., Sparks, MD, USA) in a proliferation medium (HAM F12, Sigma, supplemented with 10% horse serum and 10% FCS) and kept in a 5% CO_2 atmosphere at 37 °C. After 3 days in culture, the medium was exchanged to Dulbecco's Modified Eagle's Medium (Sigma) supplemented with 4% horse serum to facilitate myoblast fusion and differentiation. Experiments were carried out on 4–8 days old cultures.

Cells were removed from the incubator and kept for 30 min in normal Tyrode's solution (in mM, 137 NaCl, 5.4 KCl, 0.5 $MgCl_2$, 1.8 $CaCl_2$, 11.8 HEPES-NaOH, 1 g/l glucose, pH 7.4) at room temperature (22–24 °C) before AFP was applied at a concentration of 10 μg/ml.

2.6. *Preparation of thin brain slices*

Thin brain slices were prepared as reported previously [20]. Briefly, 7–14-day-old Wistar rats were decapitated, and 150–200 μm thick, sagittal slices of the cochlear nucleus were cut by employing a Campden vibratome (Campden Instruments, Loughborough, UK). The slices were kept in artificial cerebrospinal fluid (aCSF; in mM: NaCl, 125; KCl, 2.5; glucose, 10; NaH_2PO_4, 1.25; $NaHCO_3$, 26; $CaCl_2$, 2; $MgCl_2$, 1; myo-inositol, 3; ascorbic acid, 0.5; sodium-pyruvate, 2). The pH of the aCSF was set to 7.2, when gassed with 95%O_2/5%CO_2, and its osmolarity was 320 mosmol/l.

During the electrophysiological experiments, the slices were continuously perfused (~1 ml/min) with gassed aCSF solution. The extracellular solution contained 1 μM tetrodotoxin (TTX; Alomone Labs, Jerusalem, Israel) during the recordings, to prevent the activation of voltage-gated Na^+ channels. AFP was applied at a concentration of 10 μg/ml.

2.7. *Measurement of $[Ca^{2+}]_i$*

To introduce the calcium sensitive probe into the myoplasmic space, cells were incubated with 10 μM fluo-3 AM (Molecular Probes, Eugene, OR, USA) in the presence of 150 nM neostigmine (Pharmamagist, Budapest, Hungary) for 30 min. The fluo-3 loaded cells were then placed on the stage of an inverted fluorescence microscope (Axiovert 200, Zeiss, Oberkochen, Germany). The dye was excited at 488 nm using an argon ion laser and a confocal scanner (LSM 510, Zeiss). The emission was monitored at 510 nm. Images were either acquired in the x–y mode (512 × 512 pixels) as a time series, or in the line-scan mode (512 pixels/line, 1.54 ms/line). Images are presented as raw fluorescence after artificial colouring.

2.8. *Recording of ionic currents in neurones and astrocytes*

Measurement of ionic currents on cells of the central nervous system followed the protocol used in previous studies [7,22]. Briefly, whole-cell patch-clamp pipettes were fabricated from borosilicate glass capillaries (Clark Electromedical Instruments, Reading, UK), and filled with a solution containing (mM): KCl, 120; HEPES, 40; $MgCl_2$, 1; EGTA, 10; Na_3-GTP, 0.5; MgATP, 2. The resistance of these patch-pipettes varied between 2 and 4 MΩ. The series resistance was usually between 3 and 7 MΩ and it was compensated by 40–80%. For current recording, an Axopatch 200A patch-clamp amplifier connected to a DigiData 1200 interface (Axon Instruments, Foster City, CA) was used. Data acquisition and analysis were performed by employing the pClamp 6.0 software. Unless otherwise stated, cells were incubated with 10 μg/ml AFP for at least 5 min prior to the measurements.

2.9. *Testing for antifungal effects of AFP in the ionic milieu used for mammalian-cell assays*

Fungal growth was tested in YPG (0.3% yeast extract, 1% peptone, 2% glucose, pH 4.5), aCSF and Tyrode buffers, respectively, in 96-well flat-bottom microplates (Greiner, Kremsmuenster, Austria) inoculating each well with conidiospores in a concentration of 10^4–10^5/ml. Both aCSF and

Tyrode's solution were supplemented with 0.3% yeast extract and 1% peptone to provide suitable N-source for growing fungi. AFP was added to each well in concentrations of 1 and 10 μg/ml, and cell suspensions were incubated in a total volume of 120 μl for 48 h at 25 °C (*F. oxysporum* IfGB 39/1201) and 30 °C (*A. niger* ATCC 9029). Hyphal growth was estimated by measuring changes in optical density at 620 nm in the microplates with an EL 340 microtiter plate reader (Bio-Tek Instruments, Winooski, VT, USA).

All protocols followed the guidelines put forth by the Declaration of Helsinki and have been approved by the Institutional Ethics or Institutional Animal Care committees.

Averages were expressed as mean ± standard error (S.E.) of the mean. Significance between groups of data was assessed using Student's *t*-test.

3. Results

3.1. *Effect of AFP on growth of endothelial cells*

In order to test whether AFP has any cytotoxic effect, endothelial cells treated with different concentrations of the drug for several hours. Cytotoxicity was assessed with the colorimetric MTT assay by calculating the proportion of measured optical density of AFP-treated and control cells and then expressing the values as percent of untreated cells. Table 1 presents pooled data from four independent experiments (each carried out in triplicates) at concentrations ranging from 2 to 100 μg/ml. These results show that AFP, in the concentration range tested, had no cytotoxic effects on human umbilical vein endothelial cells. In contrast, oxidized LDL, which was ineffective at a concentration of 50 μg/ml, resulted in a 80% cytotoxicity at 500 μg/ml.

3.2. *Effect of AFP on pro-inflammatory cytokine production*

To assess the inflammatory potential of AFP, whole human blood was incubated with various concentrations of the protein and the concentrations of released IL-6 and IL-8 were measured. Lipopolysaccharides (LPS) of Gram (−) bacteria, known to cause rapid production of pro-inflammatory cytokines such as IL-6, IL-8 and TNF-α [26], were used as positive control in this experiment. LPS in a concentration of 0.1 μg/ml, caused a massive production of both cytokines

Table 1 – Lack of cytotoxicity of AFP on human umbilical vein endothelial cells

[AFP] (μg/ml)	Norm., A_{570}[a]
2	1.07 ± 0.06
5	1.05 ± 0.07
10	1.26 ± 0.06
50	1.22 ± 0.09
100	1.13 ± 0.06

[a] Absorbance in an MTT colorimetric assay normalized to that measured in the absence of the protein using umbilical vein endothelial cells (four independent experiments). None of the changes was found to be statistically significant ($p > 0.15$).

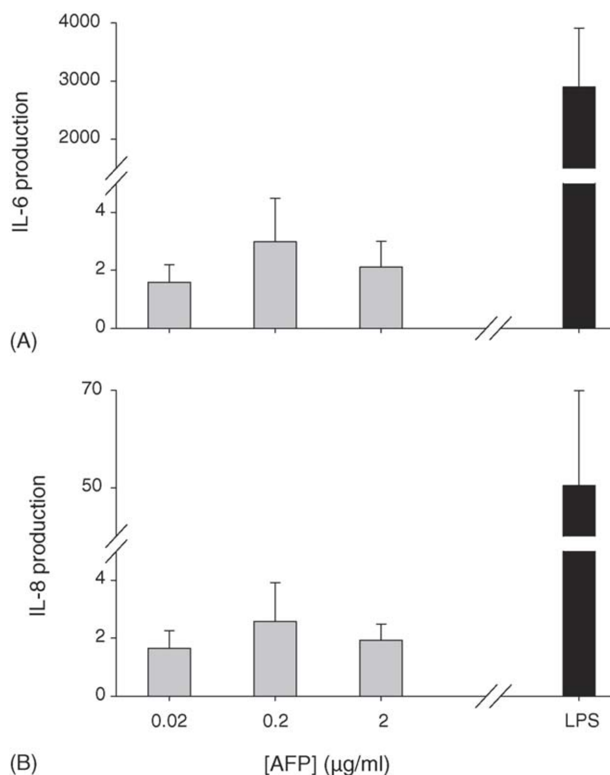

Fig. 1 – The effect of AFP on pro-inflammatory cytokine production. Different concentrations of AFP were added to blood samples from different donors and the levels of IL-6 (A) and IL-8 (B) were measured. Data were normalized to the plasma concentration of the given cytokine before the addition of AFP and are presented as averaged values from different individuals (*n* = 5). For positive control the effects of 0.1 μg/ml LPS (filled bars in each panel) were tested.

tested for each sample (Fig. 1) resulting in several 10-fold (IL-8; Fig. 1B) or thousand-fold (IL-6; Fig. 1A) increase. This established that all samples were capable of producing a large increase in cytokine production. In contrast, AFP applied at various concentrations up to 2 μg/ml failed to induce a dose dependent release of either of the two cytokines. The relative increase in cytokine production, measured in five individuals, was 1.9 ± 0.6 and 2.1 ± 0.9 for IL-6 and IL-8, respectively (Fig. 1). Based on the IL-6 response to tolerable doses of LPS (data not shown) a two- to three-fold increase in IL-6 concentrations is far below the threshold of biologically relevant responses.

3.3. *Effect of AFP on calcium homeostasis in skeletal muscle myotubes*

In order to analyze possible effects of AFP on cellular excitation and intracellular calcium homeostasis, we used skeletal muscle cells from mice in primary culture, after they have reached the myotube stage. Cells at this stage exhibited spontaneous, oscillatory contractions due to the generation of spontaneous action potentials and consequent changes in their intracellular calcium concentration ($[Ca^{2+}]_i$). These features and the well-characterized steps of excitation–contraction coupling render this preparation a useful tool

for studying the effects of potentially harmful substances on cellular excitation and intracellular calcium homeostasis. We, therefore, tested whether AFP, in a concentration of 10 μg/ml, affects the spontaneous calcium transients in fluo-3 loaded myotubes.

Fig. 2 presents images of myotubes exhibiting spontaneous elevations of $[Ca^{2+}]_i$. To visualize the differences in calcium concentration, the images were artificially coloured with blue representing lower fluorescence, and thus lower $[Ca^{2+}]_i$, whereas red representing higher fluorescence, and thus higher $[Ca^{2+}]_i$. The images were taken before (Fig. 2A) and 70 min after (Fig. 2B) the application of AFP. The presented images are two subsequent recordings from a series of thirty images taken every 800 ms. It is clearly visible from the images recorded under control conditions that certain myotubes displayed a low fluorescence in one, and a high fluorescence in the other image. Furthermore, the addition of AFP did not interfere, -not even after 70 min- with the ability of these cells to produce spontaneous calcium transients. Similar oscillatory changes in $[Ca^{2+}]_i$ were seen in all images of the series, from which the images shown in Fig. 2 were taken, as well as during the entire course of the experiment (data not shown).

To establish the time course of the calcium transients, line-scan images were recorded under control conditions and after the addition of AFP. As shown in Fig. 3, the presence of the peptide did not influence the overall features of the oscillatory changes in $[Ca^{2+}]_i$. Neither the frequency nor the amplitude of the individual transients was altered. Note that the $[Ca^{2+}]_i$ rose simultaneously over the entire cross-section of the myotubes, indicating that spontaneous action potentials rather than spontaneous uncontrolled release from the SR (sarcoplasmic reticulum) underlies the observed transients.

To further strengthen the role of spontaneous action potentials in the generation of the calcium transients and to ensure that interfering with the excitability of the cells would indeed abolish these transients, the effect of 0.3 μM tetrodotoxin was elucidated. As shown in Fig. 2A inset, this well-known blocker of the voltage-gated fast Na^+ channels readily suppressed the spontaneous calcium transients within 5 s. The fact that AFP did not influence these changes in $[Ca^{2+}]_i$ thus strongly suggests that it affects neither the excitatory processes nor the intracellular calcium homeostasis of these cells.

3.4. *Effect of AFP on potassium currents expressed by astrocytes and neurons*

A *Penicillium chrysogenum*-derived antifungal peptide (PAF) with high homology to AFP and an overlapping susceptibility range [11] has been shown to induce an efflux of potassium from the PAF-sensitive fungus *A. nidulans* [8]. Whether AFP is also able to induce such an effect in sensitive fungi is not known so far, but if this was the case in mammalian cells, it would severely hinder a potential application of AFP. Astrocytes and neurones

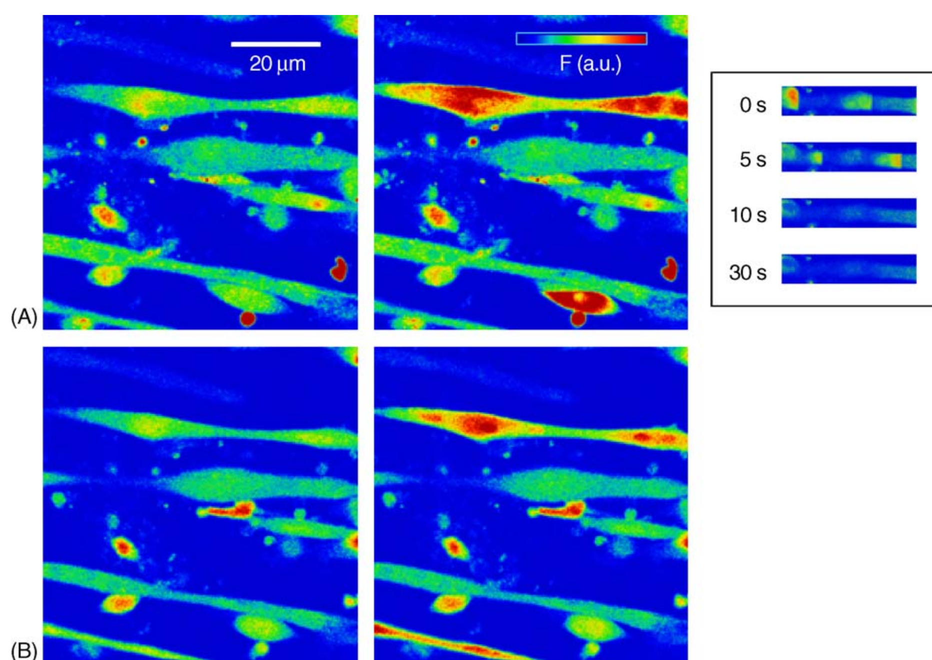

Fig. 2 – Spontaneous elevations in $[Ca^{2+}]_i$ in the absence and in the presence of AFP. *x–y* images of skeletal muscle cells maintained in primary culture before (A) and 75 min after the addition of 10 μg/ml AFP (B) to the culture medium. The images are representative examples of two series of 30 images recorded prior to and after the addition of AFP. The two images shown in panels A and B were recorded 900 ms after one another. Images were obtained by measuring the fluorescence of the calcium sensitive probe fluo-3 excited using the Argon-ion laser of a confocal laser scanning microscope. The images were artificially coloured with red representing high fluorescence, thus elevated $[Ca^{2+}]_i$, while blue representing low $[Ca^{2+}]_i$. Note that the cells were exhibiting spontaneous elevations in $[Ca^{2+}]_i$ (red areas) both under control circumstances and after a 75 min long exposure to AFP. The inset in panel A shows the effect of 0.3 μM TTX on the spontaneous calcium transients on a selected cell. Images were recorded at the indicated times, TTX was applied following the acquisition of the image at *t* = 5 s.

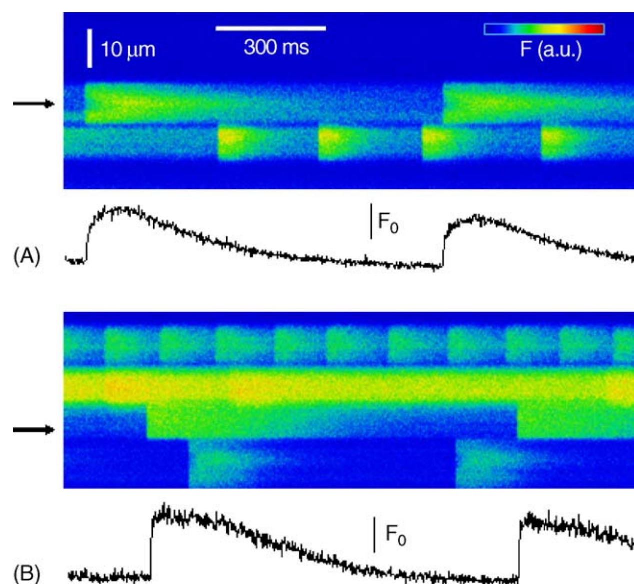

Fig. 3 – Repetitive calcium transients observed before and after the application of AFP. Line-scan images of skeletal muscle cells in primary culture before (A) and 25 min after the addition of 10 μg/ml AFP (B) to the culture medium. Images were artificially coloured as described for Fig. 2. Traces below each image represent the changes in $[Ca^{2+}]_i$, assessed as $\Delta F/F_0$, by first calculating the F/F_0 image then averaging neighboring pixels at the spatial location marked by the arrows. F_0 was determined from the area with the lowest fluorescence at a given spatial location. Note that transient rises in $[Ca^{2+}]_i$ were readily observable both in the absence and presence of AFP.

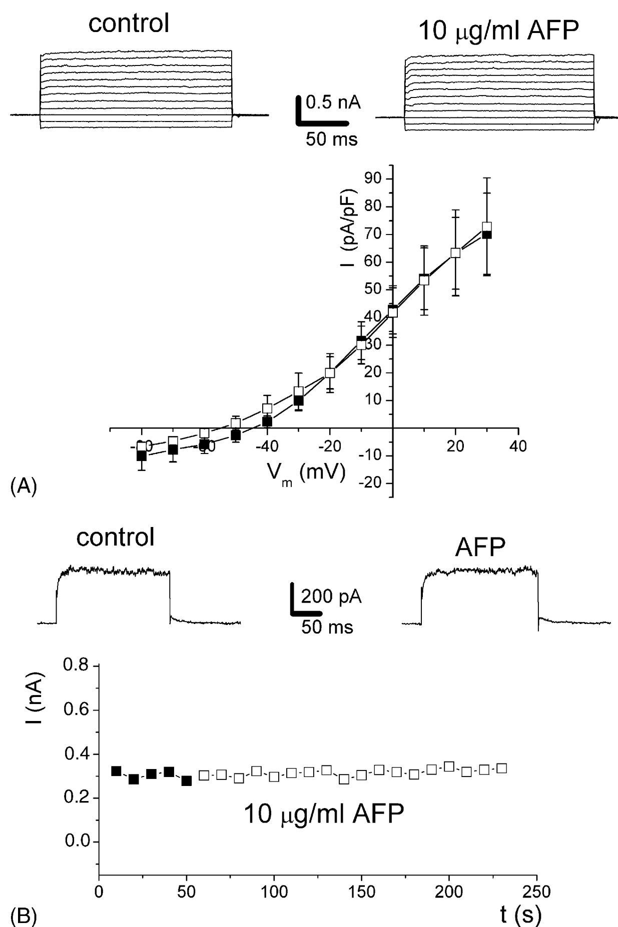

Fig. 4 – Effect of AFP application on the voltage-gated K^+ currents of astrocytes maintained in tissue culture. (A) Current traces evoked in control extracellular solution (left) and in the presence of AFP (right). During these measurements a holding potential of −60 mV was employed, and 200 ms long voltage steps were delivered from −80 to +30 mV in 10 mV steps. Bottom panel shows the synopsis of the data obtained in control solution (black squares) and in the presence of AFP (empty squares). To avoid the disturbing effect introduced by the different sizes of the astrocytes, the current amplitudes were normalized to the cell surface, and the current densities were plotted as the function of the membrane potential ($n = 9$). (B) Current traces evoked by the application of 200 ms long depolarizing pulses (to 0 mV) from a holding potential of −60 mV. The control panel was recorded prior to the application of the drug, whereas the AFP labeled panel was obtained 3 min after the initiation of the exposure to the protein (10 μg/ml). The bottom panel shows the time course of the drug application, the individual squares represent the current amplitudes of the traces recorded in 10 s intervals before (black squares) and during (empty squares) the application of AFP.

are known to express a rather diverse array of K^+ channels, thus these cells are ideal subjects of the investigation of a potential K^+ channel blocker substance. Fig. 4B demonstrates representative current traces recorded when a cultured astrocyte was depolarized to 0 mV from a holding potential of −60 mV in the absence and in the presence of AFP. As it can be clearly assessed, the protein affected neither the kinetic properties nor the amplitude of the depolarization-activated current. The time course of the application is presented beneath the current traces, documenting that the amplitude of the current did not change during the 3 min long application of the drug.

Fig. 4A shows current recordings obtained in the absence and in the presence of AFP. In this and similar experiments a holding potential of −60 mV was applied and 200 ms long depolarizing voltage steps were delivered from −80 to +30 mV in 10 mV steps. When quantifying the effect of the drug, current densities were calculated to avoid the possible disturbing effect of the different sizes of the astrocytes. The current–voltage relationship presented in Fig. 4A illustrates, that AFP did not induce statistically significant changes in the K^+ current densities of the astrocytes.

To check for the potential effect on the K^+ currents possessed by neurons, the so-called bushy cells of the cochlear nucleus were studied. Bushy cells were selected for the present experiments as they form a clearly recognizable population of neurons in the ventral cochlear nucleus whose membrane properties have been extensively studied [5]. A representative recording is shown in Fig. 5A, where 200 ms long voltage steps were delivered (between −80 and +30 mV in

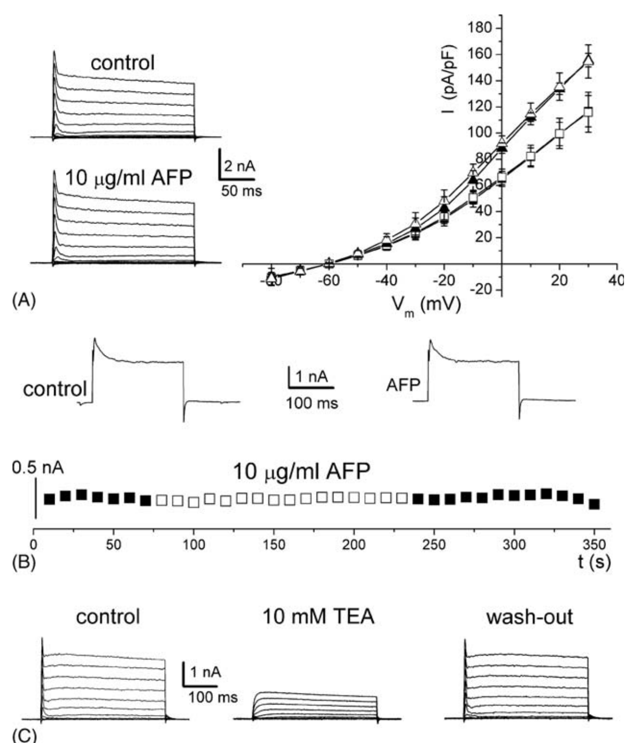

Fig. 5 – Effect of AFP on the voltage-gated K^+ currents of cochlear bushy cells in thin brain slices. (A) Current traces recorded in control conditions (top) and in the presence of AFP (bottom) from the same bushy neuron. The voltage steps were delivered from a holding potential of −60 mV, with a duration of 200 ms. The individual voltage steps followed each other in 10 mV steps between −80 and +30 mV. The panel on the right displays the current–voltage relationship of the current densities (*n* = 6) measured at the peak (triangles) and at the end of the 200 ms long pulses (squares), in control (black symbols) and in AFP containing extracellular solution (open symbols). (B) Time course of AFP application in a cochlear bushy neuron. Current traces evoked in control extracellular solution (left) and in the presence of AFP (right). During these measurements a holding potential of −60 mV was employed, and 200 ms long voltage steps were delivered to 0 mV. The bottom panel shows the time course of the drug application, the individual squares represent the current amplitudes recorded at the end of the 200 ms long stimuli. The individual pulses followed each other in 10 s intervals before (black squares), during (empty squares), and after (black squares) application of AFP. (C) Current records from a cochlear bushy neuron before, during the application and following the removal of 10 mM TEA (from left to right). Experimental conditions were the same as in (A) except for the duration of the pulses which was 400 ms.

10 mV steps) from a holding potential of −60 mV. It can be clearly seen that the presence of 10 μg/ml AFP did not alter the current traces. The current–voltage relation of the peak and the steady-state current densities are also plotted demonstrating the lack of effect on the voltage-gated K^+ currents of the bushy cells. In addition, AFP was applied for 3 min and current traces were evoked by applying depolarizing pulses to 0 mV (from a holding potential of −60 mV) in every 10 s. As illustrated in Fig. 5B, the current traces recorded prior to and after the application of AFP showed no changes at all. To establish that the currents were indeed K^+ currents the effect of tetraethylammonium (TEA), a well-known potassium channel blocker, was also investigated. As shown in Fig. 5C the presence of 10 mM TEA powerfully blocked the depolarization-activated currents at every voltage tested. The effect was reversible.

3.5. *Antifungal effects of AFP in aCSF and Tyrode's solution*

Since the antifungal activity of AFP was reported to decrease in solutions containing Na^+ [23] it was important to see if the lack of effects on mammalian cells reported above were not due to a complete loss of antifungal activity of AFP. We, therefore, tested whether AFP would have growth inhibitory effect on target fungi, *A. niger* and *F. oxysporum*, in the media used for measurements on mammalian cells. It was found that these fungi grow in these media (aCSF and Tyrode's solution) provided that they are supplemented with nitrogen sources. Under these conditions AFP inhibited the growth of both strains in a dose-dependent manner. At the concentration of 10 μg/ml (used in the experiments described above) the inhibition in aCSF was 35 ± 1% and 88 ± 0.1% while in Tyrode's solution it was 75 ± 1% and 61 ± 6% for *A. niger* and *F. oxysporum*, respectively. These data clearly establish that AFP retained its antifungal activity despite the presence of cations in the media. The lack of effect on mammalian cells should, therefore, be explained in the framework that the effect of AFP is specific for target fungi.

4. Discussion

Previous studies revealed that AFP species-specifically inhibits the growth of a variety of pathogenic fungi by binding to their outer layer and cell wall and causing severe distortions and finally permeabilization of their plasma membranes [23,25, and unpublished data]. Such membrane perturbing action, if present on mammalian cells, would greatly impair any potential therapeutic use of AFP. In this study, we therefore treated selected mammalian cells with AFP in order to investigate its potential effect on their growth and function and to evaluate whether AFP shows promise for a future application as antifungal drug in human therapy and/or plant protection. Our experiments on umbilical vein endothelial cells clearly suggested that, at least in the concentration range tested (2–100 μg/ml), AFP does not affect the growth and viability of mammalian cells. These findings are in line with our previous report where a structurally related antifungal protein derived from *Penicillium chrysogenum* (PAF) does not show cytotoxic effects on human endothelial cells isolated from the umbilical vein [22].

AFP is a protein with a molecular weight of 5.8 kDa. Although our previous experiments with the related antifungal protein PAF revealed no significant pro-inflammatory

action [22], it was of importance to confirm this for AFP as well. In this study we show that, similarly to PAF, AFP did not initiate the release of pro-inflammatory cytokines (IL-6 and IL-8). These observations suggest that these structurally related proteins are not recognized by cells of the human innate immune system and thus do not initiate an antigen-specific inflammatory response.

A direct evaluation of membrane structure in the presence of AFP was not carried out in this study. However, the observation that skeletal muscle cells in culture were not affected for as long as an hour or longer in the presence of 10 μg/ml AFP, a concentration significantly higher than the one causing detrimental membrane perturbations in sensitive fungi, suggests that mammalian cell membranes are not targeted by this protein. This is in line with the finding that neither the excitability of the cultured cells investigated here nor the voltage-gated potassium channels of neurons and astrocytes were affected by the protein. In this respect, AFP again behaved similarly to the structurally related PAF, which had no effect on the potassium currents of mammalian excitable cells. This differential effect of AFP on fungal and mammalian membranes can only be resolved if AFP either has a specific binding site on sensitive fungi or if fungal membranes are substantially different in their structure and/or composition than their mammalian counterparts. The fact that certain fungi are also resistant to AFP [23] seems to favor a specific binding site on the affected cells.

Although it has been suggested that AFP might bind to DNA since it carries an oligonucleotide–oligosaccharide binding motif [10], immunogold labeling carried out by Theis and co-workers [25] could not confirm any nuclear staining. On the other hand, a sporadic intracellular localization of AFP was observed, which does not rule out a putative intracellular target of AFP. In this respect it is important to point out that our result on the persistence of repetitive calcium transients on cultured skeletal muscle cells in the presence of AFP have clear indications: they not only suggest that AFP does not interfere with voltage-gated sodium- and calcium-channels which are believed to underlie the repetitive activity on these cells [3] but also imply that the protein does not interfere with the intracellular calcium handling of mammalian cells.

5. Conclusions

Here we report that AFP, an antifungal protein derived from *A. giganteus*, has neither cytotoxic nor pro-inflammatory effect in the potential therapeutic concentration range. Furthermore, the protein failed to alter the potassium currents of neurons and astrocytes and had no effect on the calcium oscillations of cultured skeletal muscle cells, indicating that it does not affect either the plasma membrane or the calcium homeostasis of mammalian cells. Due to the various beneficial characteristics of the peptide, such as its biological origin, the high antifungal potency, extreme physical stability, narrow host range and the lack of any significantly detrimental effects on mammalian cells, we consider in AFP an extremely attractive candidate for the future development as an antifungal agent.

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