Protein kinase A signaling and calcium ions are major players in PAF mediated toxicity against *Aspergillus niger*

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Keywords:  
Antifungal protein  
PAF  
*Aspergillus niger*  
Calcium homeostasis  
Protein kinase A signaling

1. Background

The number of newly identified small, cationic, cysteine-rich antifungal proteins that are produced by filamentous *Ascomycetes* is steadily increasing. Our knowledge about their mode of action, however, badly lacks behind, although scientists put major efforts into their characterization. This phenomenon may be based on variable degrees in similarity concerning primary sequence, solution structure, conformational dynamics, mechanistic function and antifungal spectrum [1–5]. However, most of these antifungal proteins are toxic against human-, animal- and plant pathogenic filamentous fungi, but less or not effective against bacteria or yeasts [6]. One of the best-studied antifungal proteins is PAF from *Penicillium chrysogenum* that elicits a complex response leading to severe perturbation of the calcium (Ca\(^{2+}\)) homeostasis and a sustained increase of the Ca\(^{2+}\) resting level in response to PAF [8,9].

The cyclic nucleotide cAMP and cellular Ca\(^{2+}\), both second messengers, allow the integration of information originating from multiple upstream inputs and enable quick transmission of signals through the cell. The interaction of cAMP mediated signaling and cytoplasmatic Ca\(^{2+}\) has been intensively studied in animal, plant and yeast cells [10], whereas the cross-talk and regulation of these pathways is less well understood in filamentous fungi.

PAF consists of two catalytic (PKA\(_{c}\)) and two regulatory (PKA\(_{r}\)) subunits forming a heterodimer. Upon activation, four molecules of cAMP bind to the PKA\(_{r}\) subunits resulting in the release of PKA\(_{c}\), which phosphorylates downstream targets. The PKA activity regulates most diverse cellular processes, e.g. morphology, hyphal growth, conidiation, virulence, pathogenicity and dimorphic switching [11–13].

Ca\(^{2+}\) signaling, on the other hand, is typically based on a fast and transient increase in cytosolic free Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_{c}\)). The unique...
signature of the [Ca\(^{2+}\)]\(_c\) change determines the specificity in the signaling response that regulates a wide range of processes like hyphal tip growth, branching, differentiation, cell cycle, stress response and virulence [14–16].

So far, we were able to observe the involvement of the cAMP/ PKA signaling cascade and the perturbation of the Ca\(^{2+}\) homeostasis in response to the antifungal compound PAF as independent events in two different model organisms [7,8], but the direct link between both signaling pathways was still missing. The aim of our study was therefore to investigate the cross-talk between cAMP/PKA signal transduction and the perturbation of the Ca\(^{2+}\) homeostasis in response to PAF by using one sensitive model organism.

To achieve our objective we took advantage of the elegant Aspergillus niger mutant test system previously characterized and described by [17,18]. These A. niger strains lack the catalytic subunit (strain ΔpkaC) or have a multiple copy integration of the catalytic and the regulatory subunit (mcpkacCR). The mutant mcpkacCR contains an equal copy number (10 copies) of the genes pkaC and pkaR, which are simultaneously over-expressed. The activity of PKA was reported to be 6 times higher in this mutant than in the wt control (0.6 mU/mg vs. 0.1 mU/ml, respectively). PKA activity is, however, still under control of PKA\(_R\) and cAMP [18]. In contrast, the ΔpkaC has no detectable PKA activity [18]. Importantly, these strains express the codon-optimized Ca\(^{2+}\) sensitive photo-protein aequorin for the determination of the [Ca\(^{2+}\)]\(_c\) signature in response to external stimuli [15].

Our investigations proved for the first time our hypothesis that cAMP/PKA signaling and the sustained elevation of [Ca\(^{2+}\)]\(_c\) in response to PAF treatment are interconnected and regulate PAF toxicity. Our study further underline that this mechanistic function of the antifungal protein PAF is common in sensitive fungi.

2. Materials and methods

2.1. Strains and chemicals

A. niger strains used in this study are listed in Table 1. All strains were obtained from the strain collection of the Department of Biotechnology, National Institute of Chemistry, Ljubljana, Slovenia. Chemicals were purchased from Sigma Aldrich (Austria) unless otherwise stated.

2.2. Production of PAF

PAF was purified from the supernatant of a 72 h old liquid shake culture of P. chrysogenum Q176 (ATCC 10002) as described previously [8]. For the generation of recombinant PAF (mPAF) and a mutated PAF version (PAF\(^{K35A/K38A}\)) the Pichia pastoris KM71 expression system (Invitrogen, Life Technologies, Austria) was used. Site-directed mutagenesis and cloning were performed as described in [3]. For recombinant expression of mPAF and PAF\(^{K35A/K38A}\) the manufacturer’s instruction (Invitrogen) was applied. In brief, one single colony of positively transformed P. pastoris, respectively, was used to grow a preculture in 1 L BMG (1.34% YNB, 4 × 10\(^{-5}\)% biotin, 1% glycerol, 100 mM potassium phosphate pH 6.0) at 28 °C and continuous shaking until log phase was reached (OD\(_{600}\) = 2–6). The 1 L preculture was pelleted, resuspended in 100 ml BMM (BMG with 0.5% methanol instead of 1% glycerol) and grown under the same conditions as the preculture, whereby 100% methanol was added to the culture to a final concentration of 0.5% every 24 h. After 96 h of cultivation, the supernatant was collected for protein purification as described in [3].

2.3. NMR measurements

To prove the folded structure of PAF\(^{K35A/K38A}\), \(^{1}H\) NMR was applied. Approximately 0.2 mg protein was dissolved in 450 μl 10 mM sodium phosphate buffer (pH 6.0), then 5% D\(_2\)O was added to the solution which was filled into a 5 mm diameter glass NMR tube. \(^{1}H\) NMR spectrum was obtained with water suppression using 3–9–19 pulse sequence with gradients [19]. Since protein concentration was low, 512 scans were needed for a proper spectrum with adequate signal to noise ratio. Two dimensional homonuclear \(^{1}H–^{1}H\) NOESY spectrum was acquired as well, where watergate W5 pulse sequence water suppression was applied with gradients [20]. Spectra were acquired on a Bruker Avance II 500 MHz spectrometer equipped with a 5 mm Z-gradient triple resonance probe head (Rheinstetten, Germany). Topspin 3.0 software (Bruker GmbH, Rheinstetten, Germany) was used for data acquisition, processing and plotting.

2.4. Growth inhibition assays

Antifungal activity assays were performed on appropriately supplemented solid Vogel’s medium containing PAF (0–200 μg/ml) on which 1 × 10\(^4\) conidia were dotted in 5 μl aliquots. The plates were then incubated at 30 °C for up to 72 h. Every 24 h the plates were photographed using a camera stand with the same fixed distance to the plates. Additionally, the colony diameters were determined. Activity assays with various concentrations of PAF, mPAF and PAF\(^{K35A/K38A}\) (0–200 μg/ml) were performed in liquid Vogel’s medium in 96-well plates as described previously [21]. The growth was monitored photometrically at OD\(_{620\text{nm}}\) in a microtiter plate reader (GENios Plus, Tecan, Austria) every 24 h and 48 h. All experiments were repeated at least twice.

2.5. Measurement of the [Ca\(^{2+}\)]\(_c\)

A. niger strains expressing codon optimized aequorin were inoculated at 1–5 × 10\(^5\) conidia/ml in Vogel’s medium containing 10 μM coelenterazine (Biosynth, Switzerland) and grown at 30 °C for 12 h in the dark. The calibration of [Ca\(^{2+}\)]\(_c\) and the determination of the [Ca\(^{2+}\)]\(_c\) signature were performed as described in [22] using a Microlumat LB986P plate luminometer (Berthold, Germany). All measurements were done in triplicates and repeated at least twice.

3. Results and discussion

3.1. The lack of the catalytic subunit PKA\(_C\) renders A. niger resistant to PAF

We exposed the A. niger strains with different PKA activities to increasing concentrations of PAF and determined their growth on solid medium. To this end we used the aequorin-expressing

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Table 1

| Strain | Relevant genotype | Source of reference |
|--------|-------------------|---------------------|
| wt     | Wild-type         | CBS 120.49          |
| wt\(^a\) | cspa1, aeqS, amdS\(^b\) | [17]               |
| ΔpkaC\(\) | cspa1, pyrA6, leu A1, ΔpkaC::pyrA, nicA1, aeqS | [17]               |
| ΔpkaCR | Δarg8::pyrA1, cspa1, pyrA6, leu A1, ΔpkaC::pyrA, nicA1, ΔpkaR::arg8 | [23]               |
| mcpkac | cspa1, pyrA6, leu A1, nicA1, pkaC, pyrA\(^b\) | [23]               |
| mcpkacCR\(^a\) | cspa1, pyrA6, leu A1, nicA1, pkaC::pyrA\(^b\) aeqS | [17]               |

\(^a\) Aequorin-expressing strains.
mutations ΔpkaC and mcpkACR and included also the strains ΔpkaCR and mcpkACR without aequorin expression to investigate the role of the regulatory subunit of PKA in PAF toxicity. Under control conditions (no PAF) the PKA over-expressing mutants, mcpkACR and mcPkaCR, showed similar growth and development as the wt, whereas the PKA deletion strains ΔpkaC and ΔpkaCR exhibitted smaller colony diameters compared to the wt and the mcPka mutants (Fig. 1, Table 2). Our observations matched with the phenotype description of these mutants by [8]. However, at a concentration of 200 µg/ml PAF, the ΔpkaC and ΔpkaCR strains showed a similar proliferation as the untreated controls, although asexual development was delayed (Fig. 1, Table 2).

In contrast, the radial growth of the wt strain was significantly reduced at a PAF concentration as low as 50 µg/ml (Fig. 1, Table 2). However, the mutants with elevated PKA activity, mcPkaC and mcPkaCR, were slightly less sensitive to 50 µg/ml PAF and exhibited enhanced conditiation compared to the wt. Instead, at high PAF concentrations (200 µg/ml), both multi-copy mutants were similarly susceptible to the antifungal protein as the wt, showing reduced colony diameters and delayed conditiation (Fig. 1, Table 2).

Our data therefore indicate that the lack of the catalytic subunit of PKA rescued A. niger from PAF toxicity, whereas increased PKA activity did not significantly change the fungal sensitivity to high PAF concentrations (200 µg/ml). Furthermore, neither the additional deletion nor the increased copy number of the regulatory subunits in the mutants, ΔpkaCR and mcPkaCR, respectively, resulted in any additional visible effects on the susceptibility of A. niger to PAF compared to the single mutants ΔpkaC and ΔpkaC. This indicates that PKAΔ plays a dominant role over PKAΔ in the PAF-specific response. The asexual development of the ΔpkaC and ΔpkaCR strains, however, was negatively affected by PAF and seemed not to be under the direct control of PKA activity under the test conditions applied. On the other hand, we have to note here that deregulated expression of PKA may trigger so far undefined rescue mechanisms that result in enhanced conditiation at low PAF concentrations (50 µg/ml) as observed with the mutants mcPkaC and mcPkaCR. However, at high PAF concentrations (200 µg/ml) these mechanisms may not be efficient enough to overcome the toxic effect in both mutants.

For completeness we note here that the expression of recombinant aequorin in the A. niger strains ΔpkaC and mcPkaCR did not influence the sensitivity toward PAF (Fig. 1, Table 2). This was also reflected in a comparable susceptibility of the aequorin-expressing A. niger wt and the untransformed wt strain (data not shown).

3.2. PAF triggers a specific Ca²⁺ signature in A. niger

To characterize the Ca²⁺ response to PAF, we used the aequorin-expressing A. niger wt strain. When exposing 12 h old A. niger wt germlings to PAF (0–400 µg/ml) a significant, PAF-concentration dependent and sustained elevation of the intracellular Ca²⁺ resting level could be observed (Fig. 2). The [Ca²⁺i] resting level of untreated samples was 0.04 µM (S.D. ± 0.05). When applying 400 µg/ml PAF, the [Ca²⁺i] reached 0.23 µM (S.D. ± 0.02) within the first five min before it decreased within the next five min to remain elevated at approx. 0.15 µM (S.D. ± 0.10) for the duration of measurement (30 min). Instead, the intracellular Ca²⁺ resting level of the untrearted control remained at 0.04 µM (S.D. ± 0.10). Notably, we used up to 400 µg/ml PAF to monitor the Ca²⁺ response because fungal germlings are less sensitive to PAF than conidia [8]. To further prove that the Ca²⁺ response is PAF specific, we exposed the wt strain to the protein variant PAF variant was significantly less active (Table 3). Notably, the ¹H NMR and the NOESY spectrum clearly indicated that the PAF[H35A/K38A] variant was in a folded state (Supplementary data, Fig. S1) and the structure resembled that of PAF. Therefore, unfolding of the PAF variant could be excluded to be responsible for the loss of function of PAF[H35A/K38A].

Next, we tested the effect of PAF[H35A/K38A] on the intracellular Ca²⁺ resting level of aequorin expressing A. niger germlings. In accordance to our previous observation that PAF toxicity is directly connected with the perturbation of the Ca²⁺ homeostasis, mPAF elicited a similar sustained elevation of the Ca²⁺ resting level as PAF in 12 h A. niger germlings (Table 4). In contrast, the PAF[H35A/K38A] variant failed to trigger this specific response and the [Ca²⁺i] remained at the level of the untreated control sample (Table 4). This result underlines the specificity of the PAF-elicted Ca²⁺ response and gives further evidence that the antifungal toxicity of PAF is directly connected with the perturbation of the fungal Ca²⁺ homeostasis. Furthermore, our data underline our previous suggestion that cationic motifs on the protein surface regulate the interaction of PAF with sensitive target organisms and are directly involved in mediating antifungal toxicity [3].

3.3. A. niger ΔpkaC does not respond with a PAF-specific Ca²⁺ elevation

To study the cross-talk between cAMP/PKA signaling and the Ca²⁺ response to PAF, we compared the Ca²⁺ signature in the aequorin-expressing PKA mutants ΔpkaC and mcPkaC with that of the wt. Interestingly, the mutant ΔpkaC with reduced susceptibility to PAF exhibited a Ca²⁺ resting level 3 times higher than that of the wt strain (approx. 0.14 µM vs. 0.03 µM, respectively). In this mutant, PAF failed to trigger a specific Ca²⁺ response and the [Ca²⁺i] resting level of the treated sample remained at the level of the untreated control (0.14 µM, S.D. ± 0.10) for the time of measurement with no significant relative rise in Ca²⁺ (0.7% change to control) (Table 5). In contrast, a significant increase of the Ca²⁺ resting level was triggered by PAF in the PKA over-expressing mutant mcPkaCR, although the % change was less pronounced than in the wt (+118% vs. +500% change to control, respectively) (Table 5). Nevertheless, we conclude from this finding that antifungal toxicity is mediated by the ability of PAF to evoke a significant elevation of the [Ca²⁺i] resting level.

It had been reported previously, that the Ca²⁺ channel activity is regulated by PKA-dependent phosphorylation [17]. The former characterization of the ΔpkaC mutant revealed that Ca²⁺ signaling was impaired and the [Ca²⁺i], kinetics in response to mechanical perturbation was significantly reduced [17]. This might explain, why this mutant exhibited an elevated intracellular Ca²⁺ resting level even without PAF challenge and PAF was unable to elicit a specific Ca²⁺ response. However, increased activation of Ca²⁺ channel activity in the mcPkaC mutant did not further augment the Ca²⁺ response to PAF. We therefore hypothesize that PAF might itself directly or indirectly interfere with Ca²⁺ channel activity. However, deregulation of the PKA signaling might have other/additional effects on the Ca²⁺ homeostasis and explain the differences in strain-specific susceptibility for PAF: (i) The amount and composition of specific Ca²⁺ channels/pumps/transporters of the PKA mutants might be different to the wt strain, ultimately affecting the dynamics of the Ca²⁺ response. (ii) Jerneč and Bencina [23] demonstrated the PKA-dependent regulation of the lipid biosynthesis in A. niger. PKA mutants exhibited differences in the lipid composition. The mutant lacking PKA activity had an increased content of total lipids and a 30% reduction in phospholipids, whereas the mutant with increased PKA activity showed basically a similar lipid content as the wt strain. This was suggested to affect
the permeability and fluidity of the plasma membrane and consequently to have impact on the distribution and activity of Ca$^{2+}$ channels/pumps/transporters [23]. Moreover, cell signaling may be affected since phospholipids also cover an important role as second messengers in signal transduction regulating many different cellular processes in response to environmental stimuli [23,24].

(iii) Finally, the activity of another cellular compound may modulate PKA signaling and influence/interfere with the activity of Ca$^{2+}$ channels/pumps/transporters. Some fungi contain two or more catalytic PKA subunits with overlapping or distinct functions [25,26]. In *A. nidulans* PkaBC, has a congruent as well as opposite function to PKA C in growth, conidiation and germination depending on the nutrient availability [27]. We found that the *A. niger*
signal-2+ homeostasis seems to be specific to fungal and cAMP/PKA signaling in .

interrelation between Ca2+ and cAMP/PKA signaling is required for PAFAntifungal activity and is closely connected to Ca2+ signaling. The A. niger mutant defective in cAMP/PKA signaling and Ca2+ response was more resistant toward PAF than the wt strain.

the perturbation of the Ca2+ homeostasis by antifungal proteins like PAF is a conserved mechanism common to sensitive fungi. Considering the fact that PAF is harmless for mammalian cells, in vitro [29] and in vivo [30], the reported perturbation of the Ca2+ homeostasis seems to be specific to fungal cells. The antifungal protein PAF, therefore, represents a promising molecule to develop new antifungal strategies to prevent and combat fungal infections.

Acknowledgments

We want to thank Doris Bratschun-Khan for technical assistance. The study was financed by the Austrian Science Fund (FWF, P19970 and P25894 to F.M.), the OEAD (Scientific and Technological Co-operation Austria-Slovenia, S115/2009 to M.B. and F.M.) and the Hungarian Science Fund (OTKA, ANN 110821 to G.B.).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2015.03.037.

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