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The Antifungal Activity of the *Penicillium chrysogenum* Protein PAF Disrupts Calcium Homeostasis in *Neurospora crassa*▽†

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The antifungal protein PAF from *Penicillium chrysogenum* exhibits growth-inhibitory activity against a broad range of filamentous fungi. Evidence from this study suggests that disruption of Ca^{2+} signaling/homeostasis plays an important role in the mechanistic basis of PAF as a growth inhibitor. Supplementation of the growth medium with high Ca^{2+} concentrations counteracted PAF toxicity toward PAF-sensitive molds. By using a transgenic *Neurospora crassa* strain expressing codon-optimized aequorin, PAF was found to cause a significant increase in the resting level of cytosolic free Ca^{2+} ($[Ca^{2+}]_c$). The Ca^{2+} signatures in response to stimulation by mechanical perturbation or hypo-osmotic shock were significantly changed in the presence of PAF. BAPTA [bis-(aminophenoxy)-ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid], a Ca^{2+} selective chelator, ameliorated the PAF toxicity in growth inhibition assays and counteracted PAF induced perturbation of Ca^{2+} homeostasis. These results indicate that extracellular Ca^{2+} was the major source of these PAF-induced effects. The L-type Ca^{2+} channel blocker diltiazem disrupted Ca^{2+} homeostasis in a similar manner to PAF. Diltiazem in combination with PAF acted additively in enhancing growth inhibition and accentuating the change in Ca^{2+} signatures in response to external stimuli. Notably, both PAF and diltiazem increased the $[Ca^{2+}]_c$ resting level. However, experiments with an aequorin-expressing Δ*cch-1* deletion strain of *N. crassa* indicated that the L-type Ca^{2+} channel CCH-1 was not responsible for the observed PAF-induced elevation of the $[Ca^{2+}]_c$ resting level. This study is the first demonstration of the perturbation of fungal Ca^{2+} homeostasis by an antifungal protein from a filamentous ascomycete and provides important new insights into the mode of action of PAF.

The secreted antifungal protein PAF from *Penicillium chrysogenum* is a small-molecular-mass (6.2 kDa), cationic, and cysteine-rich peptide that inhibits the growth of numerous filamentous fungi (14–16, 21). It belongs to a family of antifungal peptides which show—despite considerable amino acid homology—significant differences in species specificity and modes of action (reviewed in reference 27). Importantly, the primary structures of these antifungals show no similarity to those from higher eukaryotes, e.g., plants, insects, or mammals (see reference 28 for a detailed review on parallels with and differences between PAF and antimicrobial proteins from higher eukaryotes and their mechanisms of action). Apart from the *Aspergillus giganteus*-derived antifungal protein AFP (19, 45; reviewed in reference 29), PAF is one of the best-studied peptides of this protein family. We have shown that PAF causes rapid hyperpolarization of the plasma membrane at hyphal tips, increased K^+ efflux, induction of oxidative stress, and apoptotic cell death (21, 25) and that PAF is internalized by hyphae of PAF-sensitive fungi (33). Furthermore, we have shown that PAF interferes with at least two signaling cascades, the protein kinase C/mitogen-activated protein (MAP) kinase and the cyclic AMP (cAMP)/protein kinase A pathways, which play a role in mediating PAF toxicity (5). However, it still has to be elucidated in more detail how these PAF-dependent effects are linked.

Recent evidence indicated that the ionic strength of the growth medium interferes with the antifungal activity of PAF (21). It is possible that Ca^{2+} ions may play a major role in influencing protein toxicity in an analogous way to the Ca^{2+}-dependent mode of action of antifungal plant defensins. Supplementation of the test medium with low concentrations of $CaCl_2$ (1 to 5 mM) reversed the antifungal activity of plant defensins (34, 43–44). The defensins RsAFP2 from the seeds of *Raphanus sativus* and DmAMP1 from the seeds of *Dahlia merckii* induced K^+ efflux and Ca^{2+} uptake in *Neurospora crassa* and caused alkalinization of the growth medium (46). Another seed defensin, MsDef1 from *Medicago sativa*, was reported to cause Ca^{2+} influx and the inhibition of mammalian L-type Ca^{2+} channels, similar to the *Ustilago maydis* killer toxin KP4 (13, 40).

Ca^{2+} is a universal intracellular second messenger in eukaryotic cells (4, 36). In fungi, there is evidence for Ca^{2+} signaling regulating numerous processes, including spore germination, tip growth, hyphal branching, sporulation, infection structure differentiation, and circadian clocks, as well as responses to osmotic stress, heat shock, mechanical stimuli, oxidative stress, and electrical fields (7, 11, 17, 20, 22, 30–31, 38–39, 41, 49). Ca^{2+} signaling typically involves transient increases in intracellular Ca^{2+} concentrations originating from the extracellular

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TABLE 1. *N. crassa* strains used in this study

Strain	Relevant genotype	Source or reference
74-OR23-1A (FGSC 2489)	wt	FGSC
22A3AWTAZ6	*hygR aeqS*	50
429601510	Δ*cch-1 aeqS hygR bar*	This study

medium and/or mobilization from internal stores (4, 23, 36–37). Little was known about Ca^{2+} dynamics in filamentous fungi until a new method based on the Ca^{2+}-sensitive photoprotein aequorin was adapted, allowing routine and easy measurement of intracellular calcium dynamics in these organisms (3, 30).

The aim of our study was to investigate the effect of PAF on the level of cytosolic free Ca^{2+} ($[Ca^{2+}]_c$) in the PAF-sensitive target organism *N. crassa* to characterize its effects on stimulus-specific Ca^{2+} signatures and to define the Ca^{2+} sources responsible for the perturbation of Ca^{2+} homeostasis. By obtaining $[Ca^{2+}]_c$ measurements in living cells expressing the bioluminescent Ca^{2+} reporter aequorin, we provide novel insights into the mode of action of this biotechnologically promising antifungal protein.

MATERIALS AND METHODS

Strains, media, and chemicals. *N. crassa* strains used in this study are listed in Table 1. All strains were grown in standard Vogel's medium or in Vogel's* medium supplemented with various $CaCl_2$ concentrations but containing 10 mM instead of 37 mM KH_2PO_4 to avoid precipitation of supplemental Ca^{2+}. Control samples were treated with the respective buffers, in which PAF and the other compounds used in this study were dissolved. Unless otherwise stated, all chemicals were from Sigma (Vienna, Austria). Coelenterazine was obtained from Biosynth (Staad, Switzerland).

Purification of PAF. PAF was purified from the supernatant of 72-h liquid shake cultures of *P. chrysogenum* Q176 (ATCC 10002) as described previously (21), with minor modifications. In brief, the supernatant was cleared by centrifugation and ultrafiltration and then loaded on a carboxymethyl (CM)-Sepharose column. Eluted PAF containing fractions were pooled, dialyzed against piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES) buffer (20 mM PIPES, 1 mM $MgCl_2$, pH 6.8), concentrated in Centriprep-3 devices (Millipore, Austria), and filter sterilized. The protein concentration was determined photometrically and by SDS-PAGE and Coomassie staining.

Growth inhibition assays. Antifungal activity assays were performed in 96-well microtiter plates as described previously (21). A total of 10^3 conidia were incubated at 25°C in 200 μl of liquid medium containing various concentrations of PAF or other chemicals where appropriate. Five wells were used in parallel, and assays were carried out three times. Fungal growth (increase in biomass) was monitored spectrophotometrically (optical density at 620 nm [OD_{620}]) (Tecan Genios Plus) and microscopically after 16 h, 20 h, and 24 h of incubation. Vogel's or Vogel's* medium was used for all the assays.

Analysis of membrane permeabilization and cell viability. A combination of fluorescein diacetate and propidium iodide was used to determine whether PAF permeabilized the plasma membrane and killed conidial germlings. The assay used is that described by Palma-Guerrero et al. (35).

$[Ca^{2+}]_c$ measurement. The *N. crassa* strain expressing the codon-optimized aequorin gene (50) was grown on Vogel's slants for 7 days to achieve maximal conidiation. A total of 10^6 conidia per ml were then suspended in liquid Vogel's medium, and 5 μM coelenterazine was added. A 100-μl cell suspension was distributed to each well of a 96-well microtiter plate (Thermo Fischer, United Kingdom). Six wells were used in parallel for each treatment. The plates were incubated at 25°C for 6 h to allow conidia to germinate. To assess changes in the $[Ca^{2+}]_c$ resting level by treatment with PAF or the Ca^{2+} channel blockers diltiazem or verapamil, various concentrations of the compounds were added manually to all six wells, and measurements were started immediately. Protocols for mechanical perturbation and hypo-osmotic shock were performed as described previously (30). At the end of each experiment, the active aequorin was completely discharged by permeabilizing the cells with 10% (vol/vol) ethanol in the presence of an excess of Ca^{2+} (1.5 mM $CaCl_2$) to determine the total aequorin luminescence of each culture. Luminescence was measured with an LB 96P Microlumat Luminometer (Berthold Technologies, Germany) which was controlled by a dedicated computer running the Microsoft Windows-based Berthold WinGlow software. Conversion of luminescence (relative light units [RLU]) into $[Ca^{2+}]_c$ was done by the use of the term bert program developed by Zelter (50). Input data were converted by using the following empirically derived calibration formula: $pCa = 0.332588\ (-\log k) + 5.5593$, where k is luminescence (in RLU) s^{-1}/total luminescence (in RLU) (12). Increases in the $[Ca^{2+}]_c$ resting level were calculated by subtracting the $[Ca^{2+}]_c$ resting level of the controls from the $[Ca^{2+}]_c$ of samples treated with PAF or the Ca^{2+} channel blockers diltiazem or verapamil, respectively.

Generation of Δ*cch-1* mutant expressing codon-optimized aequorin. The *cch-1* knockout cassette was obtained from the *Neurospora* genome project (http://www.dartmouth.edu/~neurosporagenome/index.html), and we used this to delete the gene (NCU02762) and produce a homokaryotic mutant strain lacking the Δ*mus-52* mutation according to the method of Colot et al. (10). The Δ*mus-52* strain is deficient for nonhomologous end-joining and allows the generation of highly efficient gene deletions in *N. crassa* (32). Deletion of the *cch-1* gene was confirmed by Southern analysis.

For the generation of transformants with the aequorin gene, the pAZ6 plasmid used for the wild type (wt) (30, 50) was modified according to Marris (26), resulting in expression of codon-optimized aequorin with ignite selection (pAB19). The hygromycin resistance gene was excised from pAZ6 and replaced by a fragment containing a TrpC promoter, the *bar* gene for ignite resistance, and a TrpC terminator (excised from the pBARGRG1 plasmid [8]). Correct insertion of the fragment was confirmed by sequencing, and the pAB19 plasmid was further used for transformation in the Δ*cch-1* strain. Seven-day-old transformants were screened for aequorin expression, and those expressing aequorin were used for homokaryon purification by repeated plating.

RESULTS

PAF inhibits mycelial growth of *N. crassa* in a Ca^{2+}-dependent manner. Exposure of conidia of the *N. crassa* wild type (wt) to various concentrations of PAF (0.5 to 100 μg/ml) had no inhibitory effect on conidial germination but resulted in a severe inhibition of hyphal elongation. The degree of growth inhibition correlated with the PAF concentration applied (Fig. 1). The presence of 1 μg/ml PAF or 10 μg/ml PAF in the

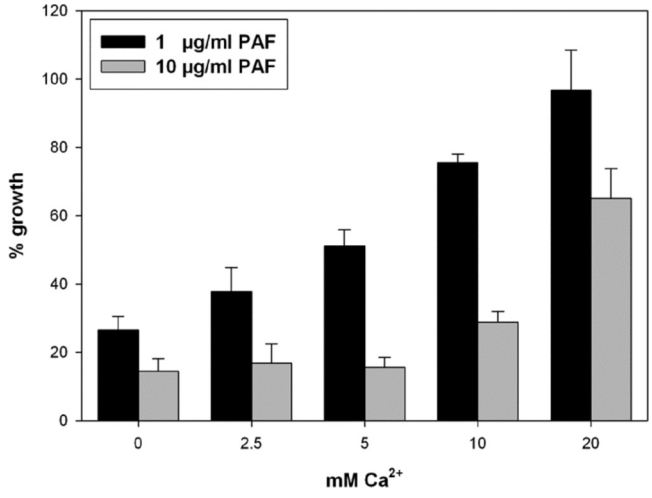

FIG. 1. Growth of *N. crassa* wt after 24 h of incubation in Vogel's* medium containing 1 μg/ml or 10 μg/ml PAF and supplemented with increasing concentrations of $CaCl_2$. Percent values were calculated from percent changes in OD_{620} values of PAF-treated *N. crassa* cells compared to untreated controls (set at 100%). Results are expressed as mean ± SD ($n = 5$).

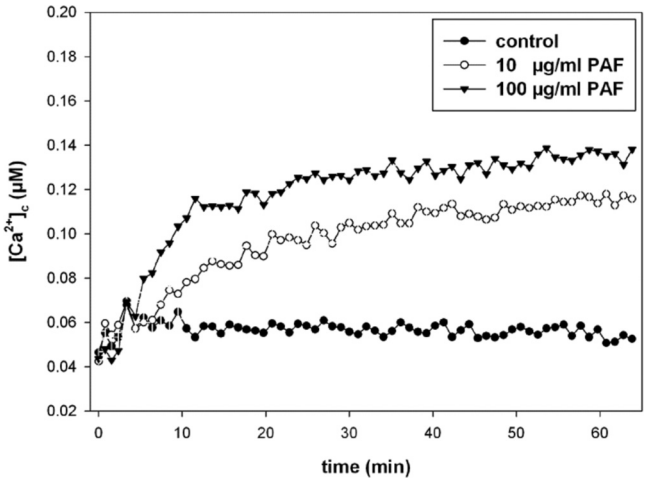

FIG. 2. Increase in resting $[Ca^{2+}]_c$ of 6-h-old *N. crassa* germlings treated with 10 μg/ml or 100 μg/ml PAF. Before protein application, the $[Ca^{2+}]_c$ resting level was determined for all wells by obtaining the average of three measurements of $[Ca^{2+}]_c$ over a 2-min period. PAF was then added, and measurements were taken every minute. Untreated samples served as controls. The SD ($n = 6$) was less than 10% of the values presented.

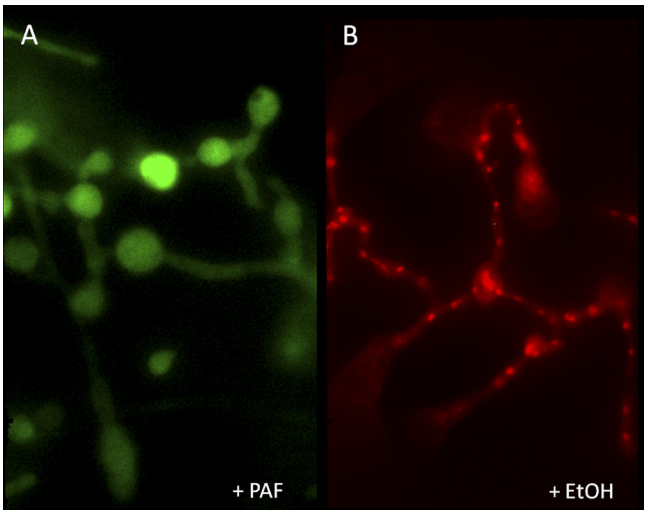

FIG. 3. Viability staining of networks of fused *N. crassa* (6 h old) germlings with fluorescein diacetate and propidium iodide. (A) The germlings were treated for 12 min (this was the time of pretreatment used prior to mechanical stimulation in the experiments involving aequorin-based calcium measurement) with (100 μg/ml) PAF in the presence of fluorescein diacetate and propidium iodide. The fungal cells are all alive because they are fluorescing green (fluorescein diacetate detects living cells), and there is no red fluorescence evident (propidium iodide is taken up into the cell only when the plasma membrane has been permeabilized). (B) Control in which a network of germlings was pretreated with (70%) ethanol (EtOH) for 40 min and stained with fluorescein diacetate and propidium iodide. Ethanol permeabilizes the plasma membrane and kills the cells. All of the germlings fluoresce red with propidium iodide, and no green fluorescence is evident.

culture medium reduced growth to 27% (standard deviation [SD]±4%) and 15% (±3%), respectively, of that of the controls. The protein concentration required for 50% growth inhibition (IC_{50}) was ~0.5 μg/ml (data not shown), and PAF at this concentration did not significantly inhibit conidial germination.

We showed previously that the toxicity of PAF is strongly influenced by the ionic strength of the test medium (21). Similarly, the activity of several other antifungal proteins (e.g., plant defensins) has been reported to be reduced in high-ionic-strength medium. Importantly, Ca^{2+} ions seem to play a major role in modulating the antifungal protein toxicity (43–44, 46). Therefore, we tested the effect of Ca^{2+} ions on PAF activity in *N. crassa*. In the absence of PAF, conidial germination and hyphal growth of *N. crassa* was the same in Vogel's medium as in Vogel's* medium supplemented with different concentrations of $CaCl_2$ (data not shown). However, as shown in Fig. 1, as little as 5 mM $CaCl_2$ significantly reduced the activity of 1 μg/ml PAF and resulted in more growth than in the samples not supplemented with $CaCl_2$. Addition of 20 mM $CaCl_2$ neutralized the activity of 1 μg/ml PAF and also reduced the activity of 10 μg/ml PAF by ~60%. This indicated that PAF might indeed interfere with $[Ca^{2+}]_c$ homeostasis in *N. crassa*.

PAF increases $[Ca^{2+}]_c$ without permeabilizing the plasma membrane. We investigated the possible influence of PAF on the $[Ca^{2+}]_c$ resting level in the wt *N. crassa* strain expressing the Ca^{2+}-sensitive photoprotein aequorin. Hyphal growth in this strain was inhibited to the same extent by PAF as was the untransformed wt strain (data not shown). $[Ca^{2+}]_c$ measurements were obtained from 6-h-old germlings every minute over a period of 60 min after treatment with various concentrations of PAF. PAF-treated samples were compared with the untreated controls (Fig. 2).

We detected a rapid and dose-dependent increase in $[Ca^{2+}]_c$ immediately after PAF application. The $[Ca^{2+}]_c$ level increased steadily from ~0.05 μM (SD, ±0.01) (baseline) within the first 10 min of measurement, to reach a maximum concentration after 60 min of 0.09 μM (SD, ±0.01) with 10 μg/ml PAF and 0.12 μM (SD, ±0.01) with 100 μg/ml PAF, respectively (Fig. 2). Untreated control samples did not show any significant change in their resting $[Ca^{2+}]_c$ levels during the 60-min period of measurement. The $[Ca^{2+}]_c$ resting level in the untreated control before treatment was 0.05 μM (SD, ±0.01) $[Ca^{2+}]_c$ and persisted over the 60-min period. In contrast, the mean $[Ca^{2+}]_c$ resting level at 55 (±5) min following PAF addition was increased 1.8-fold in the samples treated with 10 μg/ml PAF and 2.4-fold for the samples exposed to 100 μg/ml PAF compared with the untreated controls. Our results indicate that PAF evokes a significant increase in the $[Ca^{2+}]_c$ level over a 60-min period of prolonged exposure.

To test whether the PAF-induced increase in $[Ca^{2+}]_c$ involves permeabilization of the plasma membrane, we examined the effects of PAF on germlings in the presence of the membrane-impermeant propidium iodide in combination with the membrane-permeant fluorescein diacetate (35). The germlings were found not to take up propidium iodide and to retain their viability after treatment with 100 μg/ml PAF (Fig. 3A). However, in a control in which the conidial germlings were permeabilized by killing them with ethanol, the cells then stained with propidium iodide but not fluorescein diacetate (Fig. 3B).

External Ca^{2+} interferes with the PAF-induced Ca^{2+} signature. Since we showed in the growth assay that the presence of

TABLE 2. Effect of high $CaCl_2$ concentrations on the PAF-induced Ca^{2+} signature measured within the last 10 min of PAF treatment

$[CaCl_2]$ in Vogel's* medium	Relative rise in $[Ca^{2+}]_c$ (μM) (% change) with[a]:	
	10 μg/ml PAF	100 μg/ml PAF
0.7 mM	0.061 ± 0.004 (+48)	0.099 ± 0.003 (+141)
20 mM	0.002 ± 0.001 (+0)	0.002 ± 0.001 (+0)

[a] Concentrations were determined at 50 to 60 min after PAF treatment (Fig. 2). Six-hour-old germlings were pretreated with 20 mM $CaCl_2$ for 5 min before exposure to PAF. The relative rise in $[Ca^{2+}]_c$ was determined by subtracting the average $[Ca^{2+}]_c$ of the controls from the average $[Ca^{2+}]_c$ of samples treated with 10 μg/ml and 100 μg/ml PAF in the presence of 0.7 mM or 20 mM $CaCl_2$. The average $[Ca^{2+}]_c$ of the controls was normalized to 100% to evaluate the percent change in $[Ca^{2+}]_c$ of protein-treated samples. Values are means ± SDs.

20 mM $CaCl_2$ counteracted PAF toxicity (Fig. 1), we investigated the effect of externally added Ca^{2+} on the PAF-induced Ca^{2+} signature shown in Fig. 2. Prior to doing these experiments, we found that the reduction of the KH_2PO_4 concentration in the Vogel's* medium did not influence this Ca^{2+} signature compared to the fungus grown in unsupplemented Vogel's medium (data not shown). Germlings grown for 6 h in Vogel's* medium were preincubated with 20 mM $CaCl_2$ for 5 min before they were treated with PAF as described above. Cells grown in Vogel's* medium without added $CaCl_2$ were used as a control. $CaCl_2$ addition completely neutralized the PAF-specific $[Ca^{2+}]_c$ response.

This change in the $[Ca^{2+}]_c$ is summarized in Table 2 in terms of the relative rise of Ca^{2+} in the fungal cells. This was calculated by using the average $[Ca^{2+}]_c$ determined within the last 10 min of PAF treatment (50 to 60 min) and subtracting the average $[Ca^{2+}]_c$ of the controls from the average $[Ca^{2+}]_c$ of PAF-treated samples in the presence of 0.7 mM $CaCl_2$ or 20 mM $CaCl_2$. The average of the $[Ca^{2+}]_c$ of the controls was 0.041 μM (SD, ±0.003) in the presence of 0.7 mM $CaCl_2$ and 0.049 μM (SD, ±0.002) in the presence of 20 mM $CaCl_2$, respectively. No rise of $[Ca^{2+}]_c$ in high-Ca^{2+} medium was detectable when samples were exposed to 10 μg/ml and 100 μg/ml PAF, whereas increases of 48% and 141%, respectively, were reached in the absence of additional Ca^{2+} (0.7 mM $CaCl_2$). From these results it is evident that externally added Ca^{2+} indeed counteracts the effect of PAF. Thus, the neutralizing effect of Ca^{2+} ions on the growth-inhibitory activity of the antifungal protein is at least in part related to a reduced increase in $[Ca^{2+}]_c$.

PAF decreases the amplitude of the $[Ca^{2+}]_c$ response to external stimuli. It is known that a range of external stimuli transiently increase $[Ca^{2+}]_c$ levels in *Aspergillus* and *Neurospora* (3, 30). Two of these physiological stimuli are mechanical perturbation and hypo-osmotic shock, which each produces a transient increase in $[Ca^{2+}]_c$ with a unique Ca^{2+} signature, suggesting that each involves different components of the Ca^{2+} signaling/homeostatic machinery. The effects of different chemical compounds on the Ca^{2+} signatures resulting from these two stimuli can give insights into their possible influence on these different components. In our study, the characteristic Ca^{2+} signatures caused by mechanical perturbation (addition of 100 μl of Vogel's medium which is isotonic to the medium the fungus is growing in) and hypo-osmotic shock (addition of 100 μl of 10% Vogel's medium which is hypotonic to the medium the fungus is growing in) in germlings preincubated with PAF for 60 min were compared with controls that were not exposed to PAF. The $[Ca^{2+}]_c$ resting levels, kinetics, and amplitudes associated with the two different stimuli were measured. One characteristic feature of PAF-treated cells was that the $[Ca^{2+}]_c$ remained at an elevated level even after the stimulus-specific transient increase in $[Ca^{2+}]_c$ was over (Fig. 4A and B). The histogram in Fig. 4C shows the effects of mechanical perturbation and hypo-osmotic shock on the amplitude of PAF-treated samples. The amplitude of the $[Ca^{2+}]_c$ response to mechanical perturbation was reduced by 33% (SD, ±14%) with 10 μg/ml PAF and 49% (SD, ±13%) with 100 μg/ml PAF applied for 60 min before stimulus application (Fig. 4C). Sixty minutes of PAF treatment before stimulation by hypo-osmotic shock resulted in a reduction of the $[Ca^{2+}]_c$ amplitude by 26% (SD, ±8%) with 10 μg/ml PAF and 51% (SD, ±9%) with 100 μg/ml PAF.

Calcium chelator abrogates the PAF-induced rise in $[Ca^{2+}]_c$ and counteracts PAF toxicity. Extracellular Ca^{2+} and/or Ca^{2+} from intracellular Ca^{2+} stores, such as mitochondria, vacuoles, endoplasmic reticulum, and the Golgi compartment, could potentially contribute to the increased $[Ca^{2+}]_c$ in response to PAF treatment (4, 36). To determine the source of the PAF-induced increase in $[Ca^{2+}]_c$, we tested the influence of the Ca^{2+}-selective membrane-impermeable chelator bis-(aminophenoxy)-ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA). On its own, BAPTA did not influence the resting level of $[Ca^{2+}]_c$ in the conidial germlings. A 10-min pretreatment of the samples with 10 mM BAPTA before the addition of 100 μg/ml PAF inhibited the PAF-specific increase in the $[Ca^{2+}]_c$ resting level (Fig. 5A). This indicates that the PAF-dependent elevation in the $[Ca^{2+}]_c$ resting level requires the influx of extracellular Ca^{2+}.

We also found that the elevated $[Ca^{2+}]_c$ in response to a 10-min PAF treatment (100 μg/ml) dropped to the resting level immediately upon the addition of 10 mM BAPTA (Fig. 5A). We therefore conclude that extracellular Ca^{2+} is needed to execute the PAF-dependent increase in the $[Ca^{2+}]_c$ resting level that results in the perturbation of $[Ca^{2+}]_c$ homeostasis within the germlings.

The neutralizing effect of BAPTA on the PAF-induced $[Ca^{2+}]_c$ perturbation was also reflected by a reduction of the growth-inhibitory activity of PAF in the presence of BAPTA in the culture medium. Preliminary experiments showed that BAPTA inhibited mycelial growth at concentrations of >1 mM (data not shown). Therefore, 1 mM BAPTA was used in this experimental setup. In the presence of 1 mM BAPTA, the growth-inhibitory activity of 1 μg/ml PAF was slightly, but significantly, reduced (data not shown). When 10 μg/ml PAF was applied in combination with 1 mM BAPTA, the inhibitory activity of PAF was further decreased, resulting in 25% (SD, ±5%) more growth than in samples exposed to PAF alone (Fig. 5B). These results indicate that depletion of the extracellular Ca^{2+} from the growth medium ameliorates hyphal proliferation in the presence of PAF.

L-type channel blockers modulate the PAF-induced Ca^{2+} signature. To identify the source of the PAF-dependent rise in $[Ca^{2+}]_c$ in more detail and to gain a better insight into the potential role of specific Ca^{2+} transport systems involved in

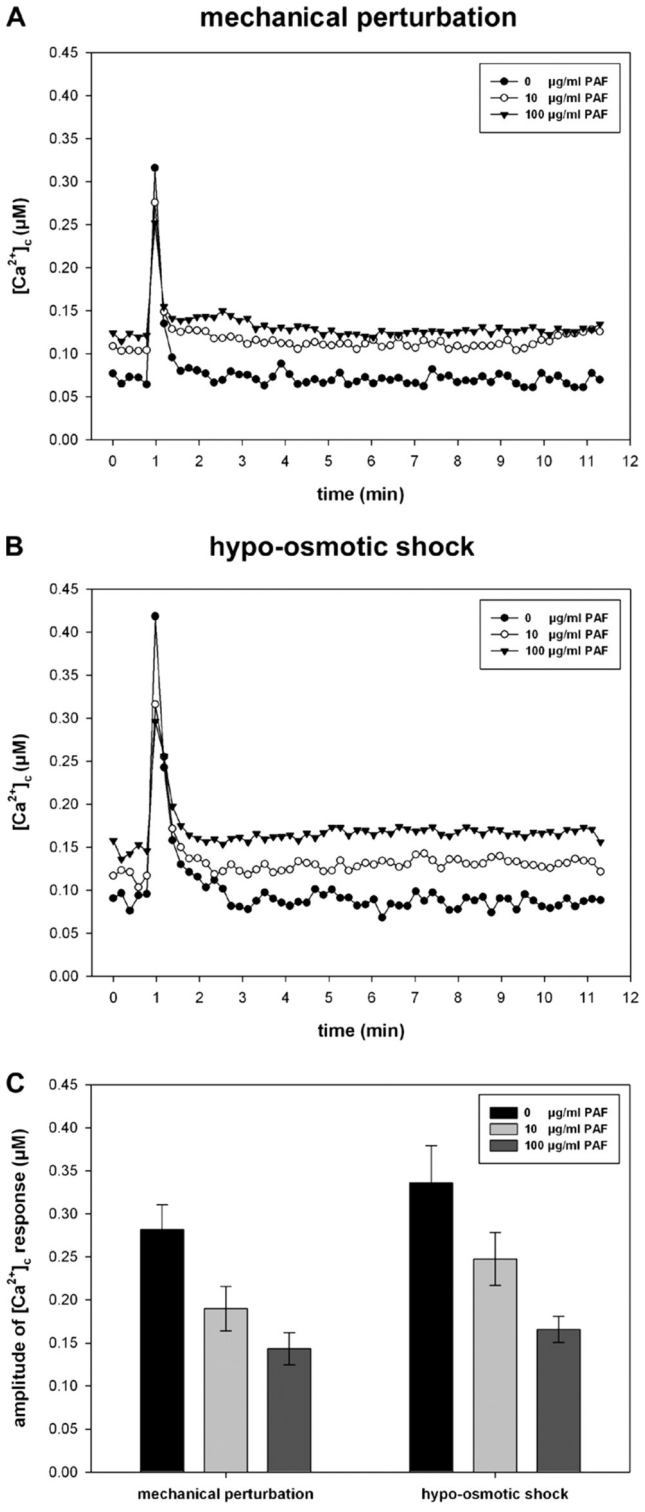

FIG. 4. Effects of PAF on the $[Ca^{2+}]_c$ responses to mechanical perturbation (A) and hypo-osmotic shock (B). Six-hour-old *N. crassa* germlings were treated with 10 μg/ml or 100 μg/ml PAF for 60 min before stimulation by mechanical perturbation (addition of 100 μl of Vogel's medium) or hypo-osmotic shock (addition of 5% Vogel's medium). The $[Ca^{2+}]_c$ signature was monitored for 12 min. The SD (n = 6) was less than 10% of the values presented. The histogram in panel C shows in detail the effects of a 60-min pretreatment with 10 μg/ml and 100 μg/ml PAF on the amplitude of the $[Ca^{2+}]_c$ responses to mechanical perturbation and hypo-osmotic shock.

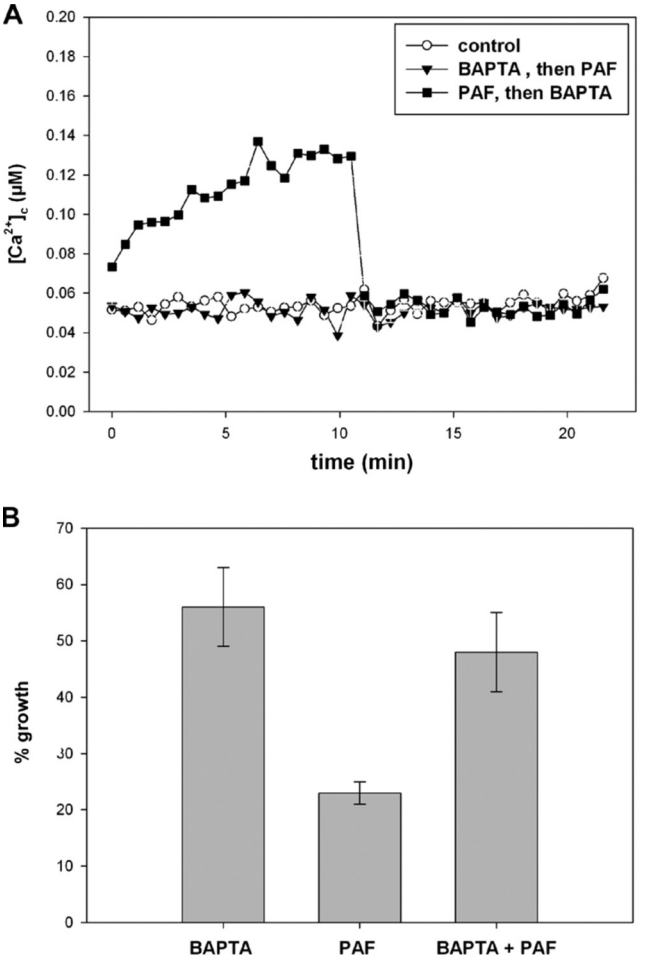

FIG. 5. Effect of the extracellular chelator BAPTA on the $[Ca^{2+}]_c$ resting level (A) and hyphal growth in the presence or absence of PAF (B). (A) BAPTA (10 mM final concentration) was applied 10 min before treatment with 100 μg/ml PAF (■) or after 10 min of PAF treatment (▼). Samples without supplements were used as controls (○). The SD (n = 6) was less than 10% of the values presented. (B) Conidia of *N. crassa* were incubated with 10 μg/ml PAF, 1 mM BAPTA, or both chemicals combined. Percent values were calculated from percent changes in OD_{620} values of PAF-treated *N. crassa* compared with untreated controls (set as 100%). Results are expressed as means ± SDs (n = 5).

PAF activity, the effects of the channel blockers diltiazem and verapamil on the response of cells with and without PAF treatment were analyzed. Both diltiazem and verapamil block the opening of voltage-gated L-type Ca^{2+} channels in animal cells, but their modes of action may be different in fungi (40, 42).

When added on its own, 0.5 mM diltiazem unexpectedly caused an increase in the $[Ca^{2+}]_c$ resting level (Fig. 6A) from 0.05 mM (SD, ±0.005) in the untreated control to 0.08 μM (SD, ±0.005) 10 min after application. PAF alone at 100 μg/ml increased the $[Ca^{2+}]_c$ resting level to 0.10 μM (SD, ±0.009) (Fig. 6A). A combination of both substances acted additively and further increased the $[Ca^{2+}]_c$ resting level to 0.15 μM (SD, ±0.01) (Fig. 6A). Verapamil at a concentration of 0.25 mM evoked similar effects (data not shown).

The addition of 10 mM BAPTA after a 15-min pretreatment with PAF, diltiazem, or both compounds combined resulted in

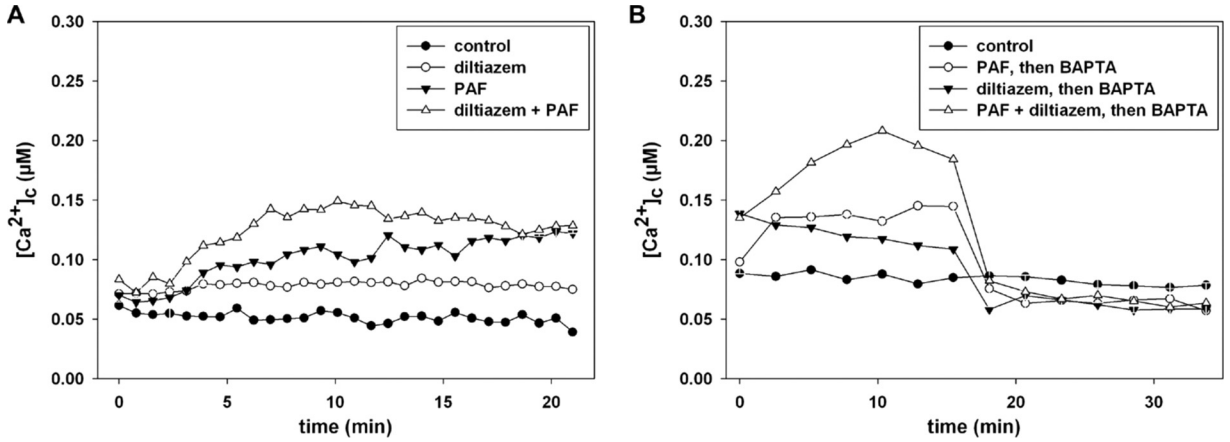

FIG. 6. Effects of the L-type channel blocker diltiazem and PAF on the $[Ca^{2+}]_c$ resting level. (A) Time-dependent effects on the $[Ca^{2+}]_c$ resting level after the substances were added at time zero. (B) The effect of the extracellular chelator BAPTA on germlings pretreated for 15 min with PAF, diltiazem, or both substances combined. In each experiment, final concentrations of 100 μg/ml PAF, 0.5 mM diltiazem, and 10 mM BAPTA were used. Untreated germlings were used as controls. Values represent averages of six wells; the SD was less than 10%.

a rapid decrease in the artificially elevated $[Ca^{2+}]_c$ resting level (Fig. 6B). This supports our conclusion that the Ca^{2+} source responsible for an elevated $[Ca^{2+}]_c$ resting level following PAF or diltiazem treatment is mainly extracellular.

The influence on the amplitude of the $[Ca^{2+}]_c$ increase in response to mechanical perturbation of diltiazem alone or in combination with PAF was also analyzed. When germlings were preincubated for 60 min with the respective chemicals, the following was observed: 0.5 mM diltiazem on its own significantly reduced the $[Ca^{2+}]_c$ amplitude from 0.36 μM (SD, ±0.03) $[Ca^{2+}]_c$ (untreated control) to 0.29 μM (SD, ±0.05), while 100 μg/ml PAF reduced the $[Ca^{2+}]_c$ amplitude to 0.21 μM (SD, ±0.05). This inhibitory effect was accentuated when PAF and diltiazem were applied together and resulted in an even lower amplitude of 0.13 μM (SD, ±0.02) $[Ca^{2+}]_c$.

The L-type channel blocker diltiazem accentuates PAF toxicity. The perturbation of the $[Ca^{2+}]_c$ signature by PAF and/or L-type channel blockers was also reflected by an additive toxicity against *N. crassa* when both substances were combined in growth inhibition assays. When subinhibitory concentrations of diltiazem (0.5 mM) or verapamil (0.25 mM) were combined with PAF (IC_{50}, 0.5 μg/ml) the growth of *N. crassa* was reduced to 43% (SD, ±1%) and 45% (SD, ±5%), respectively. This was further accentuated when higher concentrations of the compound were applied. Table 3 shows that in the presence of a normal concentration of Ca^{2+} in the growth medium (0.7 mM), the addition of 1 μg/ml PAF or 1 mM diltiazem resulted in 34% (SD, ±9%) or in 27% (SD, ±4.7%) growth, respectively, whereas a combination of both chemicals further reduced growth to only 2.2% (SD, ±0.2%) compared with the untreated controls (100%). Importantly, the additive effect of PAF and diltiazem on growth inhibition could be counteracted by supplementing the growth medium with $CaCl_2$. At 20 mM $CaCl_2$, growth was completely restored to ~100% under all three culture conditions (Table 3). These results suggest that PAF and diltiazem might have similar modes of action in *N. crassa*.

TABLE 3. Effect of high external $CaCl_2$ concentrations (20 mM) on the growth-inhibitory effect of PAF, diltiazem, and the combination of the two compounds

$[CaCl_2]$ in Vogel's* medium	% Growth of control (mean ± SD) with[a]:		
	PAF (1 μg/ml)	Diltiazem (1 mM)	PAF + diltiazem
0.7 mM	34 ± 9	27 ± 4.7	2.2 ± 0.2
20 mM	112 ± 8	110 ± 26	101 ± 18

[a] The OD_{620} was measured after 22 h of incubation. The growth of the controls was normalized to 100% to evaluate the percent growth of samples exposed to the growth-inhibitory substances.

PAF does not seem to target the putative L-type Ca^{2+} channel CCH-1. The L-type Ca^{2+} channels are one of the five main types of mammalian, voltage-gated Ca^{2+} channels (47). The Cch1 protein in the budding yeast *Saccharomyces cerevisiae* is a homologue of the pore-forming α_1 subunit of mammalian voltage-gated Ca^{2+} channels and acts cooperatively with the Mid1 protein to form a high-affinity Ca^{2+} influx system in this yeast (42). Studies by Teng et al. (42) suggested that the yeast Cch1 is pharmacologically similar to mammalian L-type voltage-gated channels because L-type channel blockers such as diltiazem and verapamil partially inhibited Ca^{2+} influx by yeast cells. In *N. crassa* an orthologous *cch-1* gene exists, but so far nothing is known about the pharmacological properties of the putative Ca^{2+} channel protein encoded.

To investigate whether the L-type Ca^{2+} channel CCH-1 might be a possible target of PAF, a *N. crassa cch-1* deletion mutant was tested for its susceptibility to PAF. The phenotype of the Δ*cch-1* strain grown on solid growth medium showed no significant difference from that of the wt strain except for a minor decrease in conidiation (data not shown). Exposure of conidia of the Δ*cch-1* strain or of a Δ*cch-1* strain expressing aequorin to various concentrations of PAF resulted in similar levels of growth inhibition as observed for the untransformed wt strain and the wt strain expressing aequorin *aeqS*.

When we tested the effect of PAF on the $[Ca^{2+}]_c$ resting level in the Δ*cch-1* strain expressing aequorin, we found that this strain responded similarly to the wt expressing aequorin (Table 4). The mean $[Ca^{2+}]_c$ level at 20 (±5) min of the

TABLE 4. Effects of PAF, diltiazem, and verapamil on the $[Ca^{2+}]_c$ resting level of the Δ*cch-1* strain expressing aequorin compared with the wt control strain expressing aequorin

Treatment	Relative rise in $[Ca^{2+}]_c$ (μM) (% change)[a]	
	wt	Δ*cch-1* strain
10 μg/ml PAF	0.043 ± 0.005 (+62)	0.040 ± 0.003 (+55)
100 μg/ml PAF	0.070 ± 0.007 (+102)	0.076 ± 0.004 (+107)
0.5 mM diltiazem	0.034 ± 0.007 (+50)	0.018 ± 0.006 (+25)
0.25 mM verapamil	0.038 ± 0.006 (+56)	0.018 ± 0.003 (+25)

[a] The relative $[Ca^{2+}]_c$ rise (measured within 20 ± 5 min) in 6-h-old germlings was determined by subtracting the average $[Ca^{2+}]_c$ of the controls from the average $[Ca^{2+}]_c$ of samples challenged with the various chemicals. The average $[Ca^{2+}]_c$ of the controls was normalized to 100% to evaluate the percent change in $[Ca^{2+}]_c$ of the treated samples. Values are means ± SDs.

untreated controls was 0.078 (SD, ± 0.002) and, hence, slightly higher than in the wt strain (0.05 ± 0.01). The relative rise in intracellular $[Ca^{2+}]_c$ for samples treated with 10 μg/ml PAF was 0.040 μM for the mutant, compared to 0.043 μM in the wt strain. At 100 μg/ml PAF the Δ*cch-1* strain possessed an intracellular $[Ca^{2+}]_c$ concentration of 0.076 μM compared to 0.070 μM for the wt. Taken together, these results do not support the putative L-type Ca^{2+} channel CCH-1 being involved in the PAF-mediated perturbation of Ca^{2+} homeostasis in *N. crassa*.

L-type Ca^{2+} channel blocker sensitivity in the Δ*cch-1* strain is reduced. When the Δ*cch-1* strain expressing aequorin was exposed to the L-type Ca^{2+} channel blockers diltiazem and verapamil, the increase in the $[Ca^{2+}]_c$ resting level was significantly lower than in the wt strain. However, both of the channel blockers still induced a rise in the resting level. The mean $[Ca^{2+}]_c$ resting level at 20 (±5) min following addition of 0.5 mM diltiazem was increased 1.9-fold more for the control strain than for the Δ*cch-1*. Similar results were obtained for samples exposed to 0.25 mM verapamil. Here, the rise in the $[Ca^{2+}]_c$ resting level in the wt strain was 2-fold of the rise of the mutant (Table 4).

These results suggest that diltiazem and verapamil target the CCH-1 channel protein in *N. crassa*, and they further support our earlier results that the observed Ca^{2+} influx induced by PAF was not mediated via CCH-1.

DISCUSSION

In this study we have shown that the antifungal protein PAF inhibits growth and perturbs Ca^{2+} homeostasis/signaling in *N. crassa.* PAF increased the $[Ca^{2+}]_c$ resting level within conidial germlings, primarily as a result of the influx of extracellular Ca^{2+}. The characteristic Ca^{2+} signatures produced in response to either mechanical perturbation or hypo-osmotic shock were altered in the presence of PAF, further indicating that PAF disrupts Ca^{2+} homeostasis/signaling. Although changes in Ca^{2+} channel activity were implicated in the PAF mode of action, the L-type Ca^{2+} channel CCH-1 was shown not to be the probable target.

Exposure of *N. crassa* germlings to PAF resulted in a very rapid and sustained increase in the $[Ca^{2+}]_c$ resting level for periods of >1 h. This increased $[Ca^{2+}]_c$ resting level may result from one or more of the following: (i) PAF forms pores in the plasma membrane causing continuous Ca^{2+} influx into the cytoplasm; (ii) PAF stimulates Ca^{2+} channel activity in the plasma membrane at a continuous low level; and/or (iii) PAF continuously inhibits Ca^{2+} pumps or antiporter activity in the plasma membrane or the organelle membranes of internal Ca^{2+} stores.

Our results with the cell-impermeant dye propidium iodide showed that the fungal plasma membrane is not permeabilized upon PAF treatment. Furthermore, previous studies on *A. nidulans* have indicated that PAF causes a hyperpolarization of the hyphal plasma membrane (25). These results indicate that PAF does not permeabilize the plasma membrane by significant pore formation because this would result in membrane depolarization, as reported for other antimicrobial proteins from bacteria, insects, and humans. Our data are consistent with the previous report that two antifungal plant defensins, RsAFP2 and DmAMP1, also evoke enhanced Ca^{2+} uptake (46).

The genomes of *N. crassa* and *Aspergillus* spp. have been predicted to encode three plasma membrane Ca^{2+} channel proteins: CCH-1, MID-1, and FIG-1 (2–3, 51). CCH-1 and MID-1 are believed to work together to produce a high-affinity Ca^{2+} influx system that is believed to provide the main Ca^{2+} entry channel in the budding yeast plasma membrane (18). Furthermore, of the three predicted plasma membrane Ca^{2+} channels in *N. crassa*, CCH-1 is the only one that is voltage gated. It is a homologue of the pore-forming α_1 subunit of L-type mammalian voltage-gated Ca^{2+} channels (2) and thus could potentially be activated by PAF-mediated plasma membrane hyperpolarization. CCH-1 thus seemed a good candidate for a PAF target. However, the Δ*cch-1* mutant was found not to exhibit resistance to PAF with increased growth and/or reduced elevation of its $[Ca^{2+}]_c$ resting level in the presence of this antifungal protein. These results thus provide strong evidence that CCH-1 is not involved in the PAF-induced Ca^{2+} response. Further evidence that PAF does actually either directly or indirectly target a Ca^{2+} channel came from the finding that the amplitude of the rapid, transient $[Ca^{2+}]_c$ increase in response to either mechanical perturbation or hypo-osmotic shock was reduced in the presence of PAF. It is presently not known which Ca^{2+} channels are activated by these external stimuli (30).

To further test whether an L-type Ca^{2+} channel is involved in increasing the $[Ca^{2+}]_c$ resting level, we performed experiments with the L-type Ca^{2+} channel blockers, diltiazem and verapamil. Diltiazem and verapamil are benzothiazepine and phenylalkylamine drugs, respectively. Both drugs block the flow of Ca^{2+} ions through L-type Ca^{2+} channels but have different modes of action by binding to different parts of the α1 subunit of the pore-forming channel (1, 9, 24, 48). Our data showed that diltiazem and verapamil had similar effects on the $[Ca^{2+}]_c$ resting level as PAF. When combined with PAF, diltiazem accentuated the effect of PAF, both in growth inhibition assays and in causing an elevated $[Ca^{2+}]_c$ resting level. These results suggested that PAF mimics diltiazem and verapamil function, and it might be that the two chemicals have the same targets in the plasma membrane. The toxicity of both components was neutralized by the presence of high Ca^{2+} concentrations in the external medium, even when both components act additively.

Our results further indicate that diltiazem and verapamil probably keep Ca^{2+} channels open to evoke a severe Ca^{2+} influx into the fungal cell. High concentrations of diltiazem were also found to result in enhanced Ca^{2+} accumulation in *S. cerevisiae* cells. Teng et al. (42) hypothesized that a specific binding of diltiazem to the yeast L-type channel Cch1p activates Ca^{2+} entry instead of blocking it. Interestingly, only 5 to 10% of the blocker concentration was necessary to be effective in *N. crassa* compared with the amount required in *S. cerevisiae*. Nevertheless, other mechanisms of action for diltiazem and verapamil have been described which may cause the $[Ca^{2+}]_c$ resting level to be elevated but which do not involve direct interference with Ca^{2+} channel activity. Anderson et al. (1) reported that these L-type channel blockers perturb the integrity and fluidity of the plasma membrane in *Escherichia coli*, thus facilitating its permeabilization by Ca^{2+} and other ions.

The normally low $[Ca^{2+}]_c$ resting level of eukaryotic cells is achieved by the combined action of buffers, Ca^{2+} pumps, and Ca^{2+} exchangers (4). The *N. crassa* genome has been predicted to encode nine Ca^{2+} ATPases and eight Ca^{2+} exchangers (51). These transport proteins remove Ca^{2+} from the cytoplasm either into Ca^{2+} storage organelles or into the extracellular medium. A number of these Ca^{2+} pumps and exchangers are predicted to be located in the plasma membrane (6, 51), and one or more of them potentially may be targets of PAF.

Our data further demonstrate that the PAF-specific increase in $[Ca^{2+}]_c$ depends on a sustained influx of Ca^{2+} ions from the external medium. The Ca^{2+}-selective chelator BAPTA not only inhibited the PAF induced rise in $[Ca^{2+}]_c$ resting level but also significantly reduced the growth-inhibitory activity of PAF. These results provide evidence of a causal link between PAF inducing an elevated $[Ca^{2+}]_c$ resting level by acting on the plasma membrane and the perturbation of $[Ca^{2+}]_c$ homeostasis resulting in growth inhibition.

The supplementation of the growth medium with elevated $CaCl_2$ concentrations reduced the growth-inhibitory activity of PAF. A possible explanation of this is that a high Ca^{2+} concentration in the growth medium may modulate the expression of one or more Ca^{2+} channels/Ca^{2+} pumps/Ca^{2+} exchangers to allow the Ca^{2+} homeostatic machinery to better maintain a low $[Ca^{2+}]_c$ resting level when subsequently challenged with PAF. However, the addition of 20 mM $CaCl_2$ for only 5 min before the PAF stimulus completely neutralized the PAF-specific response (i.e., elevated $[Ca^{2+}]_c$). This indicates that high Ca^{2+} concentrations directly increase the activity of existing Ca^{2+} pumps/transporters to counteract the PAF-specific intracellular Ca^{2+} perturbation.

The sustained increase in the $[Ca^{2+}]_c$ resting level represents a very fast and, therefore, primary response to PAF and could explain some of the detrimental effects of this antifungal protein which finally lead to programmed cell death (25), e.g., the activation of the cAMP/PKA signaling pathway (5) and the increase of intracellular oxidative stress (21). More studies are needed to decipher the mode of action of PAF and to draw a link between the distinct signaling cascades involved in PAF toxicity. Undoubtedly, this knowledge could not only contribute to a better understanding of Ca^{2+} homeostasis in filamentous fungi but also identify potential fungal targets for the development of new antifungal drugs.

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