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Released ATP Is an Extracellular Cytotoxic Mediator in Salivary Histatin 5-Induced Killing of *Candida albicans*

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Salivary histatins (Hsts) are antifungal peptides with promise as therapeutic agents against candidiasis. Hst 5 kills the fungal pathogen *Candida albicans* via a mechanism that involves release of cellular ATP in the absence of cytolysis. Here we demonstrate that released ATP has a further role in Hst 5 killing. Incubation of the cells with ATP analogues induced cell death, and addition of the ATP scavenger apyrase to remove extracellular ATP released during Hst 5 treatment resulted in a reduction in cell killing. Experiments using anaerobically grown *C. albicans* with decreased susceptibility to Hst 5 confirmed that depletion of cellular ATP as a result of ATP efflux was not sufficient to cause cell death. In contrast to Hst-susceptible aerobic cultures, anaerobically grown cells were not killed by exogenously applied ATP. These findings established that Hst binding, subsequent entry into the cells, and ATP release precede the signal for cytotoxicity, which is mediated by extracellular ATP. In a higher-eukaryote paradigm, released ATP acts as a cytotoxic mediator by binding to membrane nucleotide P2X receptors. Based on a pharmacological profile and detection of a *C. albicans* 60-kDa membrane protein immunoreactive with antibody to $P2X_7$ receptor, we propose that released ATP in response to Hst 5 activates candidal $P2X_7$-like receptors to cause cell death.

Candida albicans is the most prevalent human fungal pathogen causing severe mucosal and systemic infections in hosts with compromised immune systems (5, 28). The toxicity of the currently used polyene antimycotic drugs and emergence of resistant candidal species to the less toxic azole-based agents have initiated a search for innate antibiotics as alternative drug therapies. Innate host defense systems are evolutionarily ancient and are characterized by production of potent antimicrobial molecules that limit infections based on their capacity to selectively discriminate pathogens from self species (21). The first-line host defense of human saliva includes many proteins with potent antibacterial and antifungal activity against resident microflora in the oral cavity.

Histatins (Hsts) are 3- to 4-kDa structurally related histidine-rich basic proteins of salivary acinar cell origin that are expressed only in humans and higher subhuman primates (30). Hsts possess in vitro antimicrobial activities, and their efficacy is highest for oral yeasts, particularly *C. albicans*. Hst 3 (32 amino acids) and Hst 5 (the N-terminal 24 amino acids of Hst 3 generated by proteolytic cleavage) are the most potent candidacidal members of the family in vitro, killing yeast and filamentous forms of *Candida* species at physiological concentrations (15 to 30 μM) (32, 44). Salivary Hsts have potential as therapeutic agents in patients with oral candidiasis, since they are potent antifungal agents while being nontoxic to humans.

The physiological activities of the naturally occurring antimicrobial peptides, such as magainins (26), cecropins (2), defensins (20, 22), and bactenecins (33), have been ascribed to their effects on the microbial cell membrane. These polypeptides adopt amphiphilic α-helical, β-sheet, or poly-L-proline II structures and can form channels and disrupt lipid bilayers. Salivary Hst 5, like other linear cationic peptides, can form α-helices. However, it has been more difficult to relate the antifungal activity of Hst 5 to the structural features of its molecule. Extensive structural and conformational analyses of Hst 5 revealed that the weak amphipathic character of the helical structure precludes spontaneous insertion into microbial membranes and direct formation of pores or ion channels across the membrane (32, 34). Furthermore, Hst 5 variants with reduced killing ability exhibited similar helical contents, suggesting that the α-helical conformation is not solely responsible for optimal candidacidal activity (42). A new insight into the potential mechanism of Hst 5 candidacidal activity came from recent studies. We reported that *C. albicans* expresses a class of functional binding sites for salivary Hst 5, Hst 3, and Hst 4 and a 67-kDa yeast Hst 5 binding protein (HstBP) (13). The discovery of a specific binding protein on this yeast suggested a basis for the killing selectivity of Hsts and their lack of toxicity to human host cells, Hst 3 and 5 binding to the fungal plasma membrane was thereafter proposed to be the first event of a temperature- and ionic strength-dependent multistep killing process involving subsequent internalization of the peptides and interaction with an intracellular target(s) (19, 45). Our approach to identify cellular targets by testing pharmacological agents for effects on Hst 5 candidacidal activity resulted in identification of three chemical uncouplers (carbonyl cyanide *m*-chlorophenylhydrazone [CCCP], dinitrophenol [DNP], and azide) that inhibited Hst 5-induced killing of *C. albicans* (23). Most importantly, this work uncovered a specific cellular effect as a consequence of Hst 5 treatment that preceded cell death. *C. albicans* exposed to physiological concentrations of Hst 5 had a drastic reduction of intracellular ATP content, as a result of efflux of cellular ATP. The major characteristic of Hst 5-induced ATP release was that it occurred while *C. albicans* cells were metabolically active and had polarized membranes, thus precluding cell lysis as a possible route by which ATP was released from the cells (23).

The intracellular role of ATP has been recognized for many years; however, under appropriate conditions many cell types release ATP in the absence of cytolysis. Neurons and platelets release ATP by exocytotic secretion, and epithelial and endo-

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thelial cells release ATP through membrane ATP transporters (10, 41). Although little is known about ATP-specific channels, members of the family of ATP binding cassette (ABC) proteins have been implicated in conductive transport of ATP (36, 38).

The finding that Hst 5 caused a release of cellular ATP in the absence of cytolysis suggested a potential mechanism for its candidacidal action. Massive loss of intracellular ATP alone may induce a series of structural, biochemical, and functional alterations sufficient to trigger irreversible necrotic cellular pathways (24). Alternatively, extracellular ATP released from *C. albicans* in response to Hst 5 might have a further physiological role in cell death. In higher eukaryotic cells extensive experimental evidence has confirmed that released purine nucleotides and nucleosides interact with cell surface receptors (purinoreceptors) (P1 receptors recognize adenosine, and P2 receptors are activated by ATP) to induce a range of biological effects (4, 15). The most striking property of the membrane nucleotide receptors is their ability to mediate cytotoxicity. Recent data have established that cells susceptible to ATP-mediated cytotoxicity express either $P2X_1$, or $P2X_7$ receptors (8, 14). Consistent with the cytotoxic nucleotide receptor model, P2X agonists (ATP analogues) caused loss of *C. albicans* viability, and the antagonists suramin and pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid prevented Hst 5-induced cell killing (23).

Here we extended the characterization of Hst 5-induced ATP release from *C. albicans* and compared it to the depletion of intracellular pools and cell killing. Experiments using anaerobically grown *C. albicans* cells established that depletion of cellular ATP, as a result of an ATP efflux, is not sufficient to cause cell death and identified extracellularly released ATP as a cytotoxic mediator in Hst 5 yeast killing. Based on pharmacological and immunological evidence for presence of $P2X_7$-like receptors in *C. albicans*, we propose that ATP released in response to Hst 5 may in turn activate yeast cytotoxic nucleotide receptors to induce cell death.

MATERIALS AND METHODS

Materials. *C. albicans* strain DS1 was isolated from the palate of a denture stomatitis patient (32), and strain 31531A was obtained from E. Rustashenko and F. Sherman, Department of Biochemistry and Biophysics, University of Rochester. Fluorescein isothiocyanate-labeled Hst 3 (FITC–Hst 3) was a gift from Brian O'Connell (National Institute of Dental and Craniofacial Research, Bethesda, Md.). Sabouraud dextrose agar and yeast extract-peptone-dextrose (YPD) media were from Difco (Detroit, Mich.); CCCP, 3′-*O*-(4-benzoylbenzoyl)-ATP (BzATP), ATPγS, 2-methylthioadenosine 5′-triphosphate (2MeSATP), ADP, AMP, adenosine, and apyrase were from Sigma. ATP^{4-} was from ICN Biomedicals (Aurora, Ohio), and adenosine 5′-β,γ-methylenetriphosphate (β,γ-MeATP) was purchased from Fluka.

Hst synthesis and purification. Hst 5 (DSHAKRHHGYKRKFHEKHHSHRGY) and Hst 3 (DSHAKRHHGYKRKFHEKHHSHRGYRSNYLYDN) were synthesized using standard solid-phase synthesis protocols and 9-fluorenylmethoxy carbonyl chemistry and purified by reversed-phase high-performance liquid chromatography as described previously (13). Biotinylation of Hst 5 (biotin–Hst 5) was performed using *N*-hydroxysuccinimidobiotin (Pierce). *N*-hydroxysuccinimidobiotin (200 mg) was dissolved in 1.5 ml of dimethylformamide and mixed with 400 mg of side chain-protected Wang resin–Hst 5-NH_2 at a molar ratio of 3:l. The coupling reaction was carried out for 4 h at room temperature with stirring. The completion of biotinylation was monitored using a Kaiser test for detection of free amino groups. After filtering, three washes with methylene chloride (50:50, vol/vol), and five washes with absolute ethanol, biotin-Hst 5 deprotection, cleavage from the dried resin, and purification by high-performance liquid chromatography were carried out as described above. This procedure resulted in a specific incorporation of one biotin molecule into the free N-terminal amino group of the Hst 5 molecule, while the ε-NH_2 groups in four lysine residues remained uncoupled. The purity of biotin-Hst 5 was assessed by sodium dodecyl sulfate–15% polyacrylamide gel electrophoresis (SDS–15% PAGE) using a Tris-Tricine electrode buffer and visualized by Western blotting with ExtrAvidin conjugated horseradish peroxidase and 4-chloro-1-naphthol (Sigma). Candidacidal bioassays verified that biotin–Hst 5 and FITC–Hst 3 retained full biological activity.

Candidacidal assay. *C. albicans* was maintained on Sabouraud dextrose agar and grown in YPD or sucrose-salts-biotin yeast synthetic medium as previously described (23). For cell growth under anaerobic conditions, *C. albicans* cells were inoculated in sucrose-salts-biotin yeast synthetic medium containing Oxyrase (Oxyrase Inc., Mansfield, Ohio) and grown at 25°C according to the manufacturer's instructions. Oxyrase in broth reduces the O_2 concentration to below 10 ppb within 30 min, removes any reintroduced oxygen, and maintains this level of anaerobiosis for more than 16 days. The antifungal activity of Hst 5 was examined by a microdilution plate assay (23) with the following modifications. Briefly, *C. albicans* cells were washed with 10 mM sodium phosphate buffer (Na_2HPO_4-NaH_2PO_4) (pH 7.4) and resuspended at 5×10^5 cells/ml. Cell suspensions were mixed with Hst 5 (31 μM) and incubated for 1.5 h at 37°C with shaking. Where indicated, apyrase (40 U/ml) was included during the 1.5-h incubation of the cells with Hst 5. Candidacidal assays using nucleotide receptor agonists were carried out for 3 h at 37°C. For experiments where cell killing was directly compared to ATP release, (*C. albicans* (10^6 cells) was incubated for 1.5 h (unless indicated otherwise) at 37°C with Hst 5 (3.9 to 61 μM) in a final volume of 110 μl. Control cultures were incubated with 10 mM phosphate buffer alone. Cell suspensions were diluted, and aliquots (500 cells) were spread onto Sabouraud dextrose agar plates and incubated for 24 h at 37°C. Candidacidal assays were performed in duplicate or triplicate. Cell survival was expressed as a percentage of the control value, and loss of viability was calculated as [1 − (colonies from Hst 5-treated cells/colonies from control cells)] × 100.

ATP bioluminescence assay. ATP levels in cultures of *C. albicans* were measured as described previously (1, 9) with the following modifications. *C. albicans* (10^6 cells) was mixed with increasing concentrations of Hst 5 for various times in a final volume of 110 μl. For extracellular ATP measurements, cells were pelleted (5,000 × *g*, 3 min), and 25 μl of the supernatant was pipetted into 210 μl of boiling TE buffer (50 mM Tris, 2 mM EDTA, pH 7.8), boiled for an additional 2 min, and stored on ice until assayed for ATP. Cell pellets were then resuspended in 1 ml of TE buffer, 10 μl of the cell suspension was diluted to 1 ml, and 50 μl (500 cells) was plated on agar to assess viability as described above. Intracellular ATP measurements were made on remaining cells (10^6) that were washed twice with TE buffer, and cell pellets were submerged in liquid nitrogen followed by the addition of 400 μl of boiling TE. The cells were boiled for 4 min, subjected to another freeze-boil cycle, and placed on ice until assayed for ATP. Extracellular and intracellular ATP levels were measured by luminometry using an ATP assay kit (Sigma) according to the manufacturer's instructions. A luciferin-luciferase assay mix (100 μl) was added to 100 μl of cell lysates or 25 μl of extracellular material in 96-well black microtiter plates (Wallac), and light emission was monitored in a 1250 LKB-Wallac luminometer. Results are expressed in bioluminescence relative light units, and ATP concentrations were determined from ATP standard curves.

Overlay assays. *C. albicans* cells were grown under air or anaerobically in medium containing Oxyrase. Cells (2×10^8 to 5×10^8) were washed with 10 mM phosphate buffer (pH 7.4) and resuspended in 100 μl of cold lysing buffer (10 mM phosphate buffer [pH 7.4], 1 mM phenylmethylsulfonyl fluoride, 1 mM EDTA, 1 μg of aprotinin per ml, 1 μg of pepstatin A per ml, 1 μg of leupeptin per ml, and 1 μg of benzamidine per ml) in tubes containing 100 μl of prechilled 0.5-mm-diameter glass-beads. Cell-breakage was achieved by vigorous vortexing in five 2-min cycles at 4°C. Cell lysates were clarified by centrifugation at 12,000 × *g* at 4°C and mixed with boiling Laemmli sample buffer. Solubilized proteins were separated by SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes for overlay assay. The membranes were blocked with 1% milk in Tris-buffered saline (TTBS) (10 mM Tris-HCl [pH 7.5], 137 mM NaCl, 0.1% Tween 20), washed, and then incubated for 2 h with 250 nM biotin–Hst 5 in binding buffer (10 mM Tris-HCl, pH 7.5). Blots were extensively washed in binding buffer and then incubated for 1 h with ExtrAvidin-peroxidase at 1:5,000 in 1% bovine serum albumin in binding buffer to visualize the reactive biotinylated proteins.

Cell fractionation. *C. albicans* cells were grown overnight in 2 1 of YPD medium at 37°C, harvested by centrifugation, and washed twice with 10 mM phosphate buffer (pH 7.4). Cells were resuspended in 35 ml of cold lysing buffer and added to a 50-ml Bead Beater chamber (Biospec, Bartlesville, Okla.) containing 25 ml of prechilled 0.5-mm-diameter glass beads. Homogenization was carried out in 10 1-min bursts at 4°C. The lysate was clarified at 20,000 × *g*. The crude membrane fraction was separated from the soluble proteins by centrifugation for 1 h at 105,000 × *g*. The membrane pellet was washed and homogenized in lysing buffer containing 1% Triton X-100. The membrane fraction and cytoplasmic fraction (the high-speed supernatant) were lyophilized and prior to electrophoresis were solubilized in Laemmli sample buffer.

Immunoblot detection of proteins. Western blot membranes were blocked in TTBS with 1% bovine serum albumin and incubated for 2 h with rabbit polyclonal anti-$P2X_7$ serum (1:1,000) or with anti-$P2X_1$ serum (1:300). Anti-$P2X_7$ serum was raised against the synthetic peptide corresponding to residues 576 to 595 of the rat $P2X_7$ protein, and anti-$P2X_1$ serum was raised against residues 382 to 399 of rat $P2X_1$ receptor (Alomone Labs, Jerusalem, Israel). Following incubation with goat anti-rabbit immunoglobulin G conjugated to horseradish peroxidase, the reactive proteins were visualized using enhanced chemiluminescence (ECL, Amersham).

Confocal fluorescence microscopy. FITC–Hst 3 was used to examine Hst entry into *C. albicans* cells that were grown under air or anaerobically in medium

containing Oxyrase. Cells (10^6) were treated for 1.5 h at 37°C with 15 μM FITC–Hst 3 in 100 μl of 10 mM phosphate buffer, pH 7.4. Where indicated, a 100-fold excess of Hst 5 was added as an unlabeled competitor. The cells were then extensively washed and concentrated, and living cells were mounted on slides with sealed coverslips. Optical sections were collected using a laser scanning confocal imaging system (MRC 1024; Bio-Rad, Richmond, Calif.) with a fluorescein excitation line (488 nm) and filter systems to visualize FITC-tagged protein. The images were taken at 0.5-μm cell sections.

RESULTS

Characterization of Hst 5-induced release of *C. albicans* ATP and cell killing. We have previously shown that Hst 5 killing of *C. albicans* is initiated with a nonlytic release of ATP, which coincided with the depletion of cellular ATP and correlated with cell death (23). We extended these results by performing detailed time course and dose dependency studies of Hst 5-induced release and depletion of intracellular ATP, while aliquots of these cells were plated on agar to assess viability. This approach allowed us to directly compare extracellular and intracellular ATP levels to the number of surviving CFU, as well as permitting us to account for variations in detected ATP by normalizing to the number of control CFU (actual cell number present during incubation). ATP release and depletion of intracellular ATP depended on the concentration of Hst 5 and the length of exposure to the cells. Incubation of *C. albicans* cells for 5 to 10 min with a relatively low concentration of Hst 5 (3.9 μM) did not result in significant killing or in release or depletion of cellular ATP compared to untreated cells (Fig. 1A). Increased time of treatment caused a gradual increase in cell killing (68% at 90 min), which corresponded to a 55-fold increase in extracellular ATP and 42% depletion of intracellular ATP (Fig. 1B and C). Within the first 5 min of treatment, Hst 5 at a physiological concentration (31 μM) caused a 17% loss of cell viability and a six fold increase in extracellular ATP. Maximum release (100-fold increase in extracellular ATP) was observed following 30 min of Hst 5 (31 μM) exposure, which corresponded to an 85% depletion of intracellular ATP and 82% loss of cell viability (Fig. 1). The level of released ATP after 90 min of incubation of the cells with 31 μM Hst 5 represented over an 80-fold increase compared to the control extracellular ATP level. This treatment resulted in a reduction of the intracellular ATP level to approximately 6% of the ATP measured in control cells and corresponded to complete cell killing as assessed by the inability of the *C. albicans* cells to form colonies.

Culture anaerobiosis protects *C. albicans* from Hst 5 killing. Recent studies demonstrated that Hst 5 killing of *C. albicans* was partially inhibited when performed in an anaerobic chamber (19). These results, together with the finding that chemical uncouplers protected *C. albicans* from Hst 5 killing (23), suggested that active mitochondrial metabolism may sensitize cells to Hst 5. We therefore tested whether anaerobically grown cells were susceptible to Hst 5. For these experiments, cells were grown for 2 days in standard medium containing the enzyme preparation Oxyrase to remove oxygen and create anaerobic conditions. Respiration measurements confirmed culture anaerobiosis, since *C. albicans* cells grown under these conditions did not consume oxygen (data not shown). Furthermore, resumption of respiration was not detected for at least 1 h after cells were washed free of Oxyrase and resuspended in air-saturated medium. *C. albicans* cells grown anaerobically exhibited about 60% (strain *DSI*) (Table 1) and 57% ± 5% (strain 3153A)($n = 3$) reductions in killing when exposed for 1.5 h to 31 μM Hst 5, compared to cells grown in air-saturated medium. This protective effect was not due to an interaction of Hst 5 with Oxyrase present in the buffer, since increased survival (61% ± 2%)($n = 3$ for strain DS1) was observed even when cells were washed of Oxyrase before exposure to Hst 5 in aerated buffer.

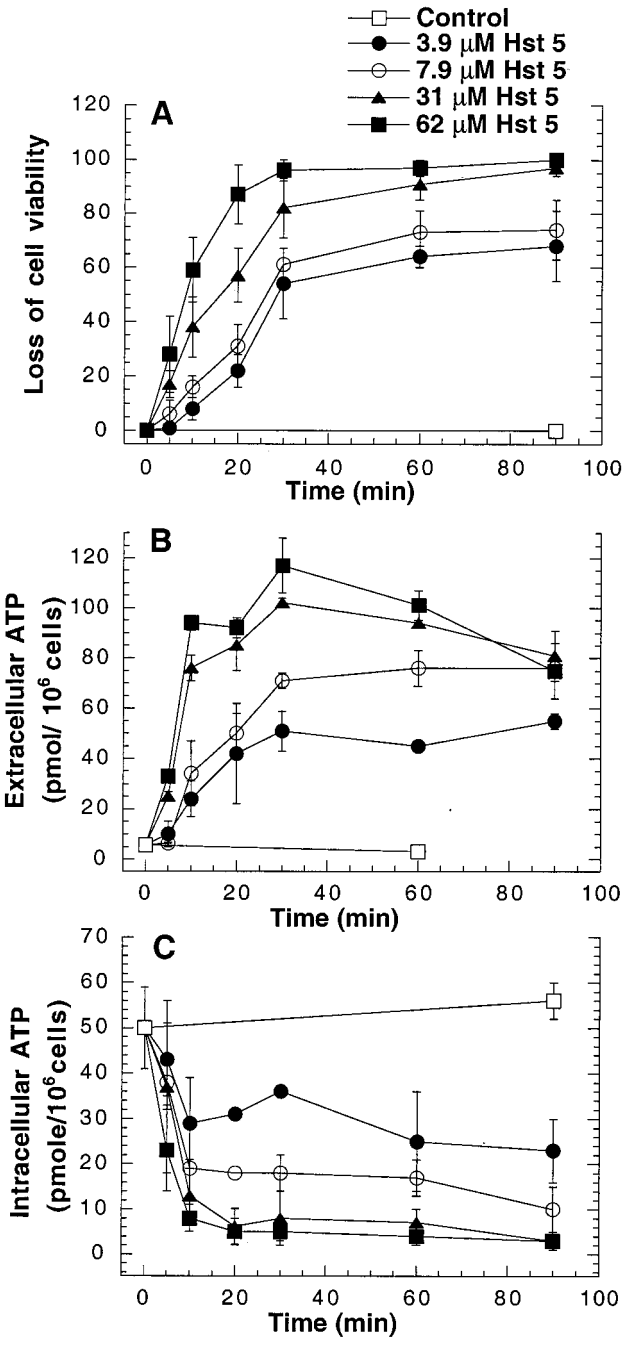

FIG. 1. Dose- and time-dependent induction of ATP release, depletion of intracellular ATP, and killing of *C. albicans* in response to Hst 5. *C. albicans* (10^6 cells, strain DS1) was incubated with increasing concentrations of Hst 5 for the indicated periods. Cell supernatants were then assayed for released ATP by luminometry (B); cell pellets were diluted and aliquots were plated on agar to assess viability (A), whereas the remaining cells were used for determination of intracellular ATP (C). ATP was measured in bioluminescence relative light units, and ATP concentrations (picomoles/10^6 cells) were determined from ATP standard curves. Loss of cell viability is expressed as [1 - (CFU of treated cells/CFU of control cells)] × 100 (the average control CFU were 480 ± 62 for cells incubated in 10 mM phosphate buffer). Results are means ± standard deviations from duplicates from three independent experiments.

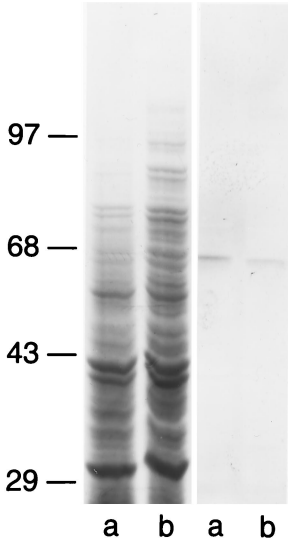

FIG. 2. Anaerobically grown *C. albicans* cells express HstBP. *C. albicans* (10^9 cells) was grown aerobically (lanes a) or anaerobically (lanes b) in synthetic medium containing Oxyrase. Proteins from whole cell lysates were separated by SDS–7.5% PAGE. Half of the gel was stained with Coomassie brilliant blue (left panel); the other half, containing identical lanes, was transferred onto a PVDF membrane and incubated for 2 h with 250 nM biotin–Hst 5 (right panel). Reactive biotinylated proteins were visualized with an ExtrAvidin-peroxidase system. The molecular masses (in kilodaltons) of protein standards are indicated to the left. The level of HstBP was not significantly altered in anaerobic cultures as assessed by densitometry scanning and normalization to unrelated proteins migrating at 40 kDa to account for loading differences.

Anaerobically grown cells express HstBP, transport Hst 5 intracellularly, and release ATP in response to Hst 5. *C. albicans* expresses functional binding sites for salivary Hsts (13, 45) and a 67-kDa candidal Hst 5 binding protein (HstBP) (13). In *Saccharomyces cerevisiae*, oxygen independent of mitochondrial respiration modulates the expression of iron uptake activities, and anaerobically grown cells lacked proteins associated with high-affinity iron uptake (18). Therefore, we examined whether growth of *C. albicans* cells in the absence of oxygen alters the level of HstBP. For these experiments, proteins from *C. albicans* cell lysates were analyzed on PVDF membranes with biotin–Hst 5. Biotin-Hst 5 bound to a 67-kDa protein (Fig. 2) that was previously recognized by ^{125}I-Hst 5 in overlay and cross-linking experiments (13). HstBP was not detected when membranes were incubated only with ExtrAvidin conjugated to peroxidase, confirming that the detected band did not represent an endogenously biotinylated *C. albicans* protein (data not shown). Overlay assays revealed that anaerobically grown *C. albicans* cells expressed HstBp at levels similar to those expressed by the susceptible aerobically grown cells (Fig. 2). Thus, the 60% reduction in killing of anaerobic cells when exposed to Hst 5 cannot be attributed to a down-regulation of HstBP.

Studies using fluorescently labeled Hst 5 or Hst 3 have shown that following binding, Hsts were transported across the yeast plasma membrane (19, 45). Therefore, we examined whether the observed reduction in killing of anaerobically grown cells reflected an inefficient uptake of Hst. *C. albicans* cells were incubated for 1.5 h with 15 μM FITC-labeled Hst 3 and examined for intracellular localization by confocal fluorescence microscopy. Both Hst-susceptible cells (aerobic cultures) and anaerobically grown cells accumulated FITC–Hst 3 intracellularly as evidenced by the punctate peripheral and discontinuous cytoplasmic pattern of fluorescence (Fig. 3). While a substantial portion of labeled Hst was clearly inside the cell and concentrated at one cell pole, the punctate staining observed on the cell surface may represent extracellularly bound protein rather than material that has been internalized. No fluorescence was observed when these cells were incubated with a 100-fold excess of unlabeled Hst 5 before the addition of FITC-Hst 3. The fact that anaerobically grown cells displayed patterns of fluorescent intracellular localization of Hst similar to those observed in cells grown under air indicates that initial Hst binding as well as its intracellular transport occur in the cells with reduced susceptibility to killing.

We next measured the ability of Hst 5 to induce ATP efflux from anaerobically grown cells, to further examine the cause of their protection against Hst 5 killing. Incubation of anaerobically grown *C. albicans* cells for 1.5 h with 31 μM Hst 5 caused a marked increase in extracellular ATP and a drastic depletion of cellullar ATP compared to the case for untreated anaerobically grown cells (Table 1). Furthermore, the effect of Hst 5 on cellular ATP in anaerobic cultures was very similar quantitatively to that observed on cells grown under air (Table 1). However, ATP efflux from aerobically grown cells resulted in complete killing, whereas despite equivalent release of cellular ATP, anaerobic growth provided about 60% protection from Hst 5-induced cell death (Table 1). Our previous studies using inhibitors of Hst 5 activity established a correlation between ATP release and killing of *C. albicans* (23). However, the present experiments with anaerobically grown cells revealed that ATP release, although required, is not sufficient for cell death.

Agonists for ATP receptors kill *C. albicans*. In higher eukaryotic cells, ATP can be transported across the plasma membrane in the absence of cytolysis through a channel-like pathway. Released ATP in turn can function outside the cell as a signaling molecule by binding to membrane nucleotide P2 receptors to cause changes in membrane permeability and even cell death (11, 43). P2 antagonists prevented Hst 5-induced killing and P2 agonists induced loss of *C. albicans* viability (23), suggesting that extracellular ATP released from the cells in response to Hst 5 may be involved in yeast killing upon activation of putative ATP receptors. We examined the ability and potency of ATP and various P2 receptor subtype-selective agonists to kill *C. albicans* (Table 2). The cytotoxic effects of BzATP ($P2X_7$ selective among P2X subtypes) and ATPγS (a P2X agonist) were detectable at concentrations lower than 0.01 μM for both ATP analogues (Fig. 4). At a concentration of 1 μM, which was the maximum extracellular concentration of ATP measured following treatment with 31 μM Hst 5 (Fig. 1), both, BzATP and ATPγS killed approximately 55% of *C. albicans* cells (Fig. 4). Increasing the concentration of BzATP, ATPγS, or ATP^{4-} ($P2X_7$-selective agonists) up to 100 μM produced about 60 to 68% cell killing (Fig. 4 and Table 2). Even at high concentrations (500 μM) 2-MeSATP (a $P2Y_1$-selective agonist), β,γ-MeATP (an agonist for $P2X_1$ and $P2X_3$), AMP, ADP, and adenosine were significantly less efficacious or ineffective (Table 2). This pharmacological profile of agonist responses is most similar to that for the $P2X_7$ cytotoxic receptor among the ATP receptor subtypes described for higher eukaryotic cells (40).

Recognition of a 60-kDa candidal membrane protein by anti-$P2X_7$ antibody. The cDNAs encoding human, rat, and mouse $P2X_7$ receptors have recently been isolated (6, 35, 40). $P2X_7$ receptors are integral membrane proteins possessing a unique cytoplasmic carboxyl-terminal domain which is required for the lytic action of ATP. We employed a specific antibody raised against the last 20 amino acids at the C terminus of rat $P2X_7$ protein to test for cross-reactivity with *C.*

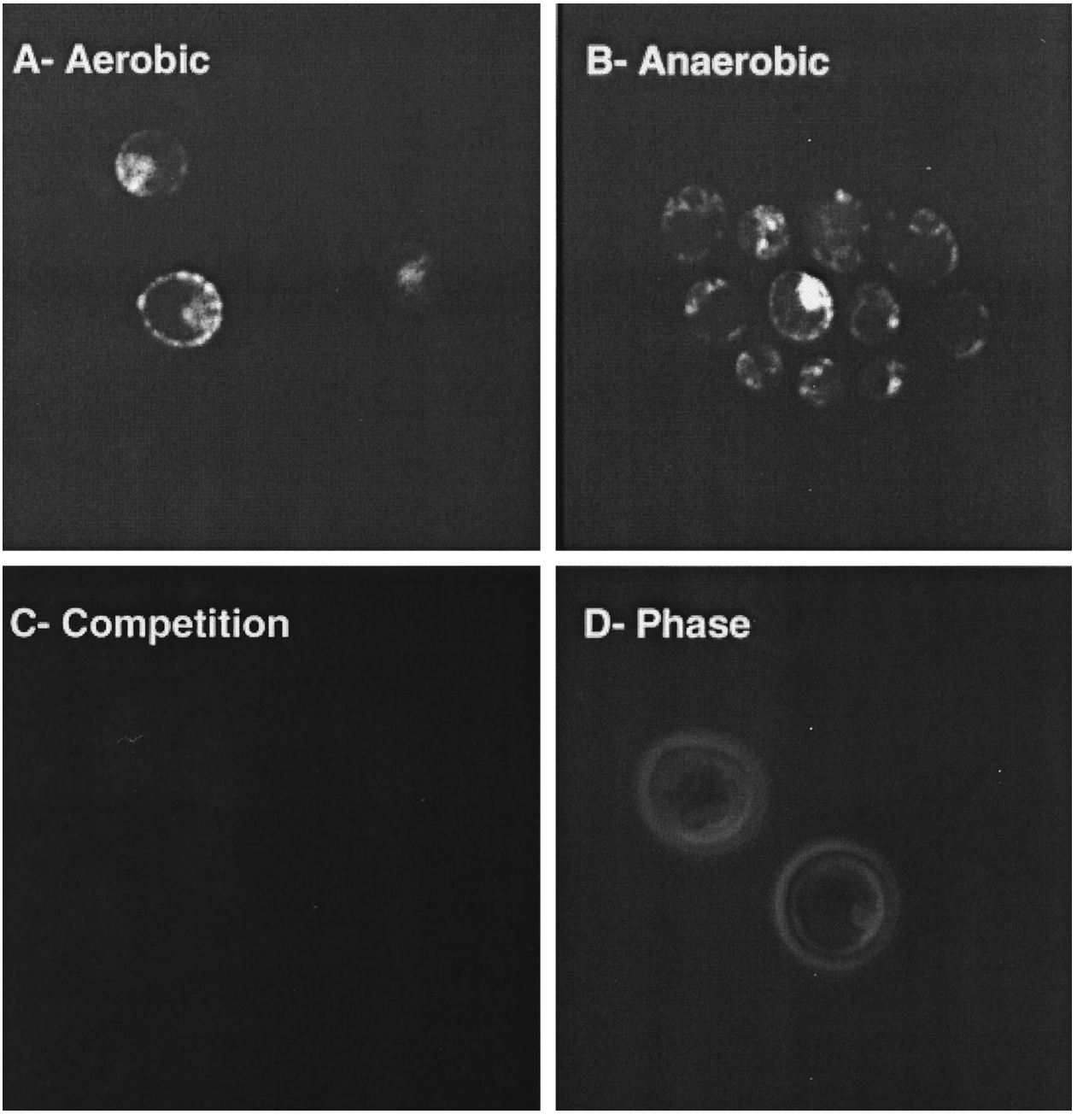

FIG. 3. Anaerobically grown *C. albicans* cells internalize Hst. Single confocal images of *C. albicans* cells (strain DS1) incubated with FITC–Hst 3 (15 μM) for 1.5 h at 37°C are shown. Aerobically grown cells (A) and anaerobically grown cells (B) display similar peripheral as well as cytoplasmic labeling by FITC–Hst 3. (C) Cells treated with FITC–Hst 3 (15 μM) in the presence of a 100-fold excess of unlabeled Hst 5. (D) Phase-contrast image of *C. albicans* cells treated with FITC–Hst 3.

albicans proteins. The anti-$P2X_7$ antibody recognized a 60-kDa protein in the membrane fractions prepared from two different *C. albicans* strains (Fig. 5A, lanes 2 and 3) but did not cross-react with yeast cytoplasmic proteins (Fig. 5A, lane 1). In control Western blots using protein lysates from J774A.1 mouse macrophage cells expressing $P2X_7$ receptors, this antibody reacted with two major bands of approximately 76 and 39 kDa (Fig. 5D, lane 2). The slower-migrating 76-kDa component is similar in size to the $P2X_7$ protein detected in ATP-susceptible microglial cells (14), and the staining of this band was completely abolished by pretreatment of the antibody with the immunizing peptide (data not shown). The immunoreactivity of the 60-kDa candidal membrane protein was greatly reduced by preadsorption of the antiserum with the cognate peptide (Fig. 6). In some experiments, a 100-kDa band and a 30-kDa band were also detected. $P2X_1$ is another ATP receptor for which a role in cell death has been proposed (8); however, antibody specific to $P2X_1$ protein did not produce a visible reaction with *C. albicans* proteins (Fig. 5B).

Extracellular ATP is a cytotoxic mediator for Hst 5 yeast killing. Although anaerobically grown cells were protected from killing, their response to Hst was indistinguishable from susceptible aerobic cultures in that they expressed HstBP, internalized Hst, and released ATP. These findings, together

TABLE 1. Anaerobically grown *C. albicans* cells release ATP in response to Hst 5 but are protected from killing[a]

Growth	Treatment	Killing (%)[b]	ATP (pmol/10^6 cells)[b]	
			Extracellular	Intracellular
Aerobic	Control	0	0.4 ± 0.2	56 ± 8
	Hst 5	97 ± 2	99 ± 34	1.1 ± 0.3
Anaerobic	Control	0	0.4 ± 0.3	49 ± 6
	Hst 5	40 ± 4	86 ± 18	4.8 ± 4

[a] *C. albicans* (10^6 cells, strain DS1) was grown aerobically or anaerobically in synthetic medium containing Oxyrase. Cells were treated for 1.5 h at 37°C with 31 μM Hst 5. Cell supernatants were then assayed for released ATP, whereas the cell pellets were used to assess viability and for determination of intracellular ATP. Extracellular and intracellular ATP levels were measured by luminometry, and loss of viability was assayed as described in the legend to Fig. 1.

[b] Values are means and standard deviations from duplicates from four independent experiments.

with the evidence for the presence of a $P2X_7$-like receptor in *C. albicans*, raised the possibility that cell protection may be due to an alteration in an ATP signaling cascade. If ATP was, in fact, a cytotoxic mediator, then exogenously applied ATP should not kill anaerobically grown cells. Consistent with this prediction, incubation of anaerobically grown cells with the P2X agonists BzATP or ATPγS at 100 μM did not result in significant killing, whereas both ATP analogues induced about a 60% decrease in survival of aerobic cultures (Hst 5-susceptible *C. albicans* cells) (Fig. 7). Thus, the inability of anaerobically grown cells to respond to extracellular ATP appears to be the cause for their reduced susceptibility to Hst 5.

The role of released ATP in Hst 5-induced cell killing was additionally supported by experiments using the phosphatase apyrase to eliminate ATP from extracellular solution. Addition of apyrase (40 U/ml) to the aerobic cells during Hst 5 treatment protected about 50% of the cells from death (Table 2). Together, our results suggest that Hst 5 binding and subsequent entry into the cells, as well as the induction of ATP release, precede the signal for cytotoxicity, which is mediated by the extracellular ATP.

TABLE 2. Pharmacological evidence for the presence of candidal cytotoxic ATP receptors[a]

Treatment	Loss of cell viability (%)[b]	
	Without Hst 5	With Hst 5
Control	0	100
ATP^{4-}	68 ± 6	
BzATP	61 ± 4	
ATPγS	62 ± 6	
2MeSATP	20 ± 2	
β,γ-MeATP	16 ± 6	
ADP	9 ± 3	
AMP	14 ± 8	
Adenosine	5 ± 3	
Apyrase	6 ± 6	54 ± 8

[a] *C. albicans* cells (strain DS1) were incubated for 3 h at 37°C with the indicated nucleotide receptor agonists (ATP^{4-}, BzATP, and ATPγS at 100 μM; 2MeSATP, β,γ-MeATP, ADP, AMP, and adenosine at 500 μM), or cells were mixed with Hst 5 (31 μM) and the ATP scavenger apyrase (40 U/ml) was included during the 1.5-h incubation at 37°C. Loss of viability was assayed as described in the legend to Fig. 1.

[b] Values are means and standard deviations of duplicate determination from seven (BzATP and ATPγS), three (2MeSATP, ADP, AMP, adenosine, and apyrase) or two (β,γ-MeATP) independent experiments.

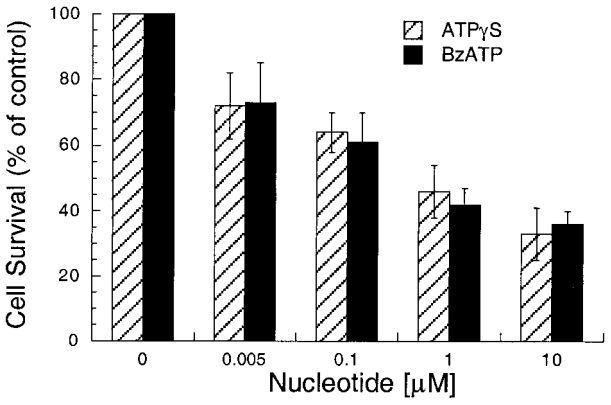

FIG. 4. BzATP and ATPγS dose-dependent killing of *C. albicans*. *C. albicans* cells (strain DS1) were incubated with increasing concentrations of nucleotide receptor agonists BzATP and ATPγS for 3 h at 37°C. Cell survival is expressed as a percentage of the control value, and each data point is the mean ± standard deviation of duplicate determinations from three to seven independent experiments.

DISCUSSION

We have previously reported that *C. albicans* cells released ATP in response to Hst 5, which occurred in the absence of cytolysis and correlated with cell death (23). In this study we showed that depletion of cellular ATP as a result of an ATP efflux is not sufficient to cause cell death and identified released ATP as an extracellular cytotoxic mediator in the Hst 5-activated killing pathway. This identification is based upon (i) the ability of extracellularly applied ATP analogues to kill *C. albicans* and (ii) the decrease in Hst-5-induced cell killing upon addition of the phosphatase apyrase to eliminate ATP from extracellular solution. Furthermore, we provide pharmacological and immunological evidence for the presence of yeast membrane ATP receptors, which may be activated by released ATP to induce cell death.

Analysis of ATP release from *C. albicans* revealed that maximum ATP efflux (0.1 ± 0.03 fmol released/cell) occurred after 30 min of incubation with concentrations of Hst 5 found in saliva (31 μM), coincided with depletion of intracellular pools (85% reduction of intracellular ATP), and correlated with cell killing (82% decrease in cell viability) (Fig. 1). The intracellular concentration of ATP in control cells was about 0.06 fmol/cell and was consistently measured using published protocols for extraction of ATP from *C. albicans* (1, 9). Comparison of the maximum level of extracellular ATP following 30 min of incubation with Hst 5 (31 μM) and the amount of ATP measured in control cells showed that more ATP was released than was available intracellularly in *C. albicans*. This difference may reflect a lower recovery of intracellular ATP due to incomplete disruption of yeast cells or hydrolysis of ATP by released cellular ATPases during the breakage of the cells. Alternatively, the detected levels of extracellular ATP may be a result of a gradual efflux of continuously synthesized ATP in the cells. This notion is supported by our previous results that production of new ATP via the mitochondrial oxidative phosphorylation in Hst 5-treated cells was carried out for at least 1 h after the maximum ATP release (23) without recovery of intracellular ATP levels (Fig. 1).

In mammalian cells ABC proteins have been implicated to function as ATP-specific channels (36, 38). *C. albicans* expresses ABC transporters that are drug efflux pumps and confer multidrug resistance (31). It is presently unknown how ATP is released from *C. albicans* in response to Hst 5 and whether

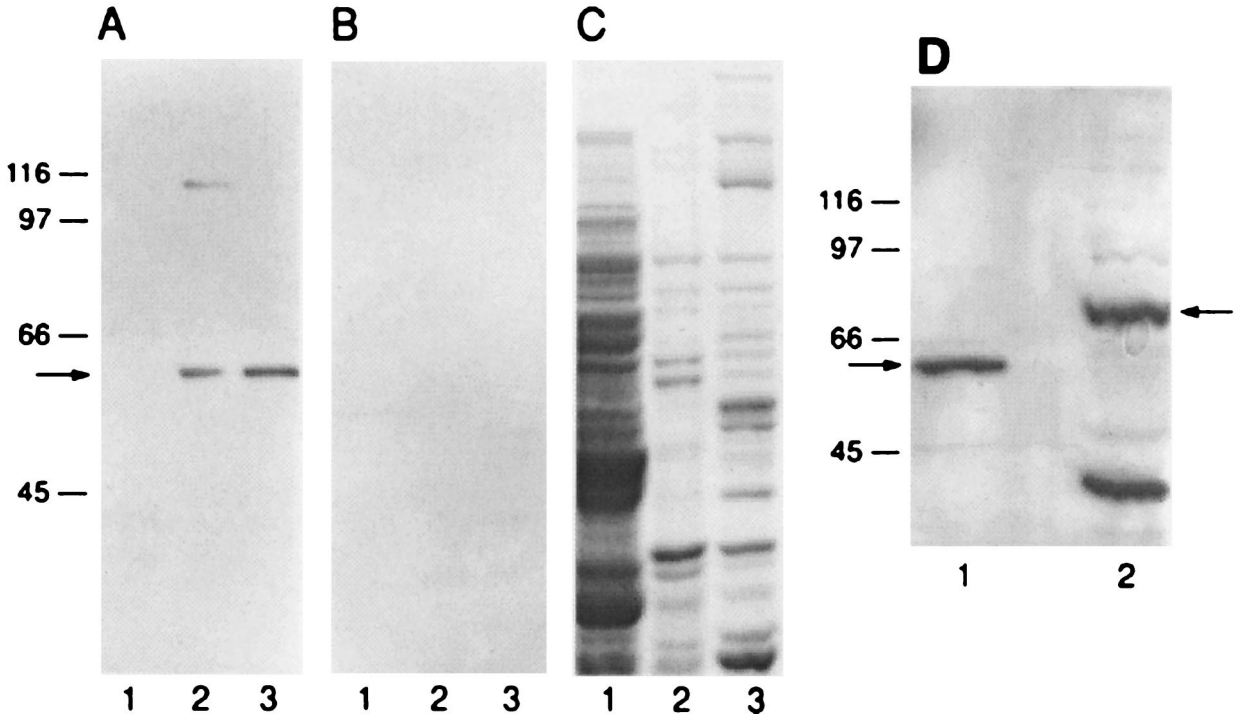

FIG. 5. A 60-kDa *C. albicans* membrane protein cross-reacts with $P2X_7$-specific antibody. (A to C) Proteins from *C. albicans* cytoplasmic (lanes 1, strain DS1) or membrane (lanes 2, strain DS1; lanes 3, strain 3153A) fractions were separated by SDS gel electrophoresis and transferred onto a PVDF membrane. The membrane was cut into three parts, each containing identical lanes, and probed with a polyclonal antiserum to the C terminus of rat $P2X_7$ receptor (1:1,000) (A) or with an antiserum to the rat $P2X_1$ receptor (1:300) (B) or were stained with Coomassie blue (C). (D) *C. albicans* membrane fraction (lane 1) and lysates from J774A.1 mouse macrophages (lane 2) were probed with anti-$P2X_7$ antibody and visualized by ECL. The arrows indicate the positions of the 60-kDa candidal membrane protein and the 76-kDa protein from J774A.1 cells recognized by the anti-$P2X_7$ antibody.

ABC proteins are involved in conductive transport; however, specific ATP release in yeast in response to toxic agents is not unprecedented (16). *S. cerevisiae* released ATP after treatment with the ionophore nigericin while the cell membrane permeability was not altered (3), and a plasma membrane ATP-specific transporter activated by the antibiotic mycobacillin was purified from *Aspergillus niger* (7).

Perhaps the most intriguing aspect of the present study concerns the functional significance of ATP efflux and the consequent depletion of *C. albicans* intracellular pools in Hst 5-induced cell death. Concentration of ATP in the cell is an indicator of viability, and gradual depletion of cellular ATP leads to inhibition of macromolecule synthesis followed by structural and functional alterations and cell death (24). Therefore, Hst 5-induced massive loss of intracellular ATP in turn may cause a series of biochemical alterations that result in the inability of the cell to replicate or initiate processes leading to later loss of cellular structure. Alternatively, extracellular ATP released from *C. albicans* in response to Hst 5 may have a further physiological role in cell death, analogous to the higher-eukaryote paradigm where released ATP acts as a cytotoxic mediator by binding to membrane nucleotide P2 receptors (12). Our studies using anaerobically grown *C. albicans* cells established that depletion of cellular ATP as a result of an ATP efflux is not sufficient to cause cell death (Table 1). Although anaerobically grown *C. albicans* cells displayed reduced susceptibility to killing, they were indistinguishable from the aerobic cultures in their response to Hst 5 in that they expressed HstBP, internalized Hst, and released ATP, which caused a severe reduction in intracellular ATP pools (Fig. 2 and 3; Table 1). Moreover, the reduction in killing was not due

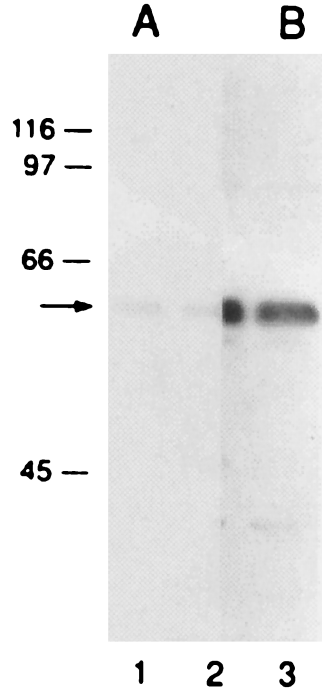

FIG. 6. Specificity of anti-$P2X_7$ immunoreactivity in *C. albicans* cells. Equal amounts of protein from the *C. albicans* membrane fraction (strain DS1) were separated and transferred to PVDF. The blot was cut in the middle, and strips were probed with $P2X_7$ receptor antibody that had been preincubated with the immunizing peptide (A) or with anti-$P2X_7$ antibody alone (B). Numbers on the left are molecular masses in kilodaltons.

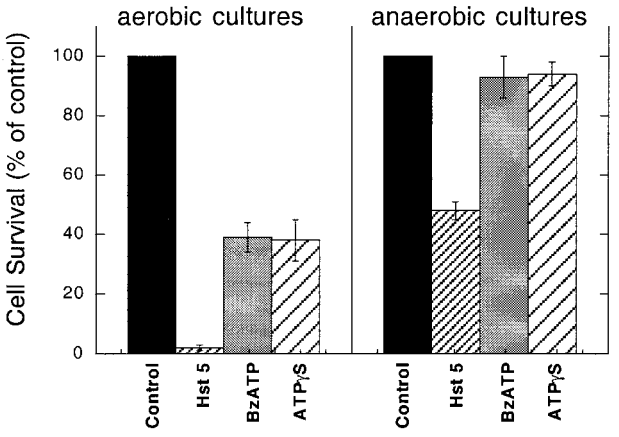

FIG. 7. Extracellular ATP is a cytotoxic agent for Hst 5-susceptible cells (aerobic cultures) but not for cells with reduced susceptibility to Hst 5 (anaerobic cultures). *C. albicans* cells (strain DS1) were grown aerobically or anaerobically as described for Fig. 2 and then incubated for 3 h at 37°C with either Hst 5 (31 μM) or BzATP or ATPγS (100 μM). In all cases cell survival is expressed as a percentage of the control value and each data point is the mean ± standard deviation of duplicate determinations from four independent experiments.

to quantitative differences in ATP concentrations, since the amount of available intracellular ATP in the untreated anaerobic cells was very similar to that in aerobically grown cells, as was the magnitude of ATP efflux and reduction of cellular ATP content following Hst 5 treatment (Table 1). The anaerobic cultures differed in their response to Hst 5 from cells grown under air in only one aspect: they did not respond to extracellular ATP, as evidenced by the inability of exogenously applied ATP analogues (BzATP or ATPγS) to kill these cells (Fig. 7). In contrast, both ATP analogues induced about a 60% decrease in survival of aerobic cultures (Hst 5-susceptible *C. albicans* cells) (Fig. 7). The functional role of released ATP in cell killing was further supported by the finding that addition of an ATP-consuming enzyme such as apyrase, to eliminate ATP from the extracellular solution during Hst 5 treatment, greatly reduced death (Table 2). Apyrase hydrolyzes ADP as well (37); however, neither ADP, AMP, nor adenosine when applied exogenously had a significant effect on *C. albicans* viability (Table 2). Together, our findings demonstrate that the cytotoxic mediator in Hst 5 killing of *C. albicans* is extracellularly released ATP.

Recent experimental evidence suggested that the candidacidal pathway activated by salivary Hsts involves at least three steps: binding, intracellular uptake, and interaction with cellular targets (19, 45). The duration of each of the steps, their contribution to killing, and when and how the cells die are not well understood. Decreased susceptibility to Hsts is a common feature of anaerobically grown *C. albicans* (Table 1), respiratory (petite) mutants (17), or cells pretreated with azide or the proton ionophores CCCP and DNP (23). In yeast, proton ionophores uncouple respiratory chain phosphorylation and induce endogenous fermentation; azide functions dually as a respiratory inhibitor and uncoupler (25, 27, 29, 39). It is notable that cells with reduced susceptibility to Hst 5 lack or have inhibited mitochondrial function. However, an interesting observation is that complete cellular protection from Hst 5 killing was not achieved by alteration of mitochondrial function (Table 1) (17, 23), suggesting that additional pathways may be involved. Furthermore, the protective mechanisms induced by culture anaerobiosis, *C. albicans* mitochondrial mutation, or drug treatment may not be the same. It is possible that inhibition of different steps in the Hst multistep killing mechanism contributes to the reduction in cell killing in these instances. Indeed, azide, CCCP, and DNP all prevented Hst 5-induced ATP efflux, resulting in an increase in cell viability (23), whereas the inability of anaerobically grown cells to respond to extracellular ATP appeared to be the cause for their reduced susceptibility to Hst 5 (Fig. 7).

Mammalian $P2X_7$ receptor is a plasma membrane ion channel and an ATP-gated pore, which can mediate ATP-induced cell death (12, 40). We have previously reported that the nonselective P2 antagonist suramin and pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid, which is relatively more selective for $P2X_7$ antagonism, prevented Hst 5-induced killing of *C. albicans* (23). The results presented here further implicate activation of *C. albicans* $P2X_7$-like receptors by released ATP in response to Hst 5 to cause cell death. Support for this model includes evidence that (i) ATP^{4-}, BzATP, and ATPγS were all capable of killing *C. albicans*, while other P2 receptor agonists were less effective or ineffective (Table 2), and (ii) an antibody specific to the unique C terminus of the $P2X_7$ protein recognized a 60-kDa candidal membrane protein (Fig. 5).

The maximum ATP concentration measured extracellularly following Hst 5 treatment (about 1 μM) resulted in nearly complete cell killing (Fig. 1), whereas exogenously applied agonists BzATP and ATPγS at 1 μM produced about 55% killing (Fig. 4). This quantitative difference in cell killing may reflect differences in the target cells themselves. In the case of Hst 5-treated cells, the released ATP acts upon cells already severely depleted of ATP, which may be more sensitive and more prone to fast ATP-mediated killing. Hst 5 entry into the cells may also activate another modulatory molecule that regulates receptor sensitivity to ATP.

The 60-kDa protein immunoreactive with antibody to $P2X_7$ was observed only in the *C. albicans* membrane fraction, however, the size of this protein is less than the apparent size of the mammalian $P2X_7$ proteins (Fig. 5). The subcellular location, together with the specific recognition by the anti-$P2X_7$ antibody but not by antibody to $P2X_1$, characterized the 60-kDa protein as a strong candidate for the candidal cytotoxic $P2X_7$-like receptor. The 67-kDa Hst binding protein, a presumptive component of the identified binding site for Hsts on *C. albicans*, was also detected in the membrane fraction (13). Preincubation of immobilized *C. albicans* proteins with anti-$P2X_7$ antibody did not decrease the binding of biotin–Hst 5 to HstBP (data not shown), thus confirming that the 60-kDa antigen and HstBP are different proteins.

The main conclusion of the present work is that extracellular ATP released in response to Hst 5 plays a role in cell killing. Collectively, our findings suggest that Hst 5 binding, subsequent entry into *C. albicans* cells, and the induction of ATP release precede the signal for cytotoxicity, which is mediated by extracellular ATP. The ability of exogenous ATP to kill *C. albicans* and the inhibition of Hst 5 cell killing by P2X antagonists (23) or upon removal of extracellular ATP (by apyrase), together with the identification of a candidal membrane $P2X_7$-like protein, support the concept that released ATP mediates killing through yeast ATP receptors.

Clearly, the candidal cytotoxic nucleotide receptor model presented above needs further experimental verification, and Hst 5 activation of other pathways leading to cell death is also acknowledged. At present, ATP receptors have been conclusively identified only in mammalian cells. There are, however, indications that ATP might have been used early in evolution as an extracellular messenger (4). Thus, in light of the therapeutic potential of Hsts in treatment of candidiasis, the pharmacological and immunological evidence for the presence of

candidal cytotoxic nucleotide receptors underscores the importance of elaborating the molecular mechanism of this previously unrecognized pathway of microbial death.

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