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RESEARCH ARTICLE

Cyclic adenosine monophosphate protects renal cell lines against amphotericin B toxicity in a PKA-independent manner

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Abstract

Amphotericin B is the "gold standard" agent in the management of serious systemic fungal infections. However, this drug can cause nephrotoxicity, which contributes up to 25% of all acute kidney injuries in critically ill patients. Cyclic adenosine monophosphate can protect kidney cells from death due to injury or drug exposure in some cases. Hence, the objective of this work was to evaluate if cAMP could prevent cell death that occurs in renal cell lines subjected to AmB treatment and, if so, to assess the involvement of PKA in the transduction of this signal. Two different renal cell lines (LLC-PK1 and MDCK) were used in this study. MTT and flow cytometry assays showed increased cell survival when cells were exposed to cAMP in a PKA-independent manner, which was confirmed by western blot. This finding suggests that cAMP (db-cAMP) may prevent cell death caused by exposure to AmB. This is the first time this effect has been identified when renal cells are exposed to AmB's nephrotoxic potential.

Keywords

cAMP, cell protection, nephrotoxicity

History

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Introduction

Avoiding renal injury is important in routine medical treatment, given that many drugs can lead to tubular damage and result in acute kidney dysfunction. Nephrotoxicity due to drug exposure contributes up to 25% of all acute kidney injuries in critically ill patients (Alvarez-Lerma et al., 2012). Amphotericin B (AmB) is categorized as a drug with a nephrotoxic potential that is still used in therapeutics because of its effectiveness. AmB has been the ''gold standard'' agent in the management of serious systemic fungal infections since the 1960s (Moen et al., 2009). Its molecule consists of a natural antibiotic from the polyene group, isolated from a strain of the actinomycete *Streptomyces nodosus* on soil collected in the Orinoco River region in Venezuela (Laniado-Laborín et al., 2009).

The AmB pharmacological mechanism of action is based on the linkage of its hydrophobic moiety to the fungal cell membrane, producing an aggregate that forms transmembrane pores (Johnson & Einstein, 2007). This leads to an increase in the permeability of membrane depolarization and proton and monovalent cations, which culminates in the disturbance of osmotic equilibrium and cell death (Sundar et al., 2014). It is possible to activate certain biochemical pathways in order to protect tissues from injuries. To effectively reduce AmB nephrotoxicity, it would be interesting to find agents that could act as protectors of cell viability.

Cyclic adenosine monophosphate (cAMP) is ubiquitous and regarded as an intracellular messenger responsible for multiple functions, including protection against cell death due to drug use in renal tissue (Qin et al., 2012). cAMP is produced by Adenylate cyclase activity which is, in turn, activated by a membrane receptor responsive to an intracellular or membrane stimulus (Dodge-Kafka et al., 2005). The signal from cAMP can be carried through ion channels, exchange protein directly activated by cAMP (Epac), and protein kinase A (PKA) (Breckler et al., 2011). PKA is a ubiquitous protein kinase expressed throughout the renal tissue, which has a wide range of functions, including cell protection (Li et al., 2014).

Thus, the objective of this work was to evaluate if cAMP could protect renal cell lines from death due to AmB exposure and, if so, to assess the involvement of PKA in the transduction of this signal.

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Materials and methods

Drugs

AmB at a purity of 90% was donated by Cristália (Produtos Químicos Farmacêuticos Ltda, Itapira, SP, Brazil). A stock solution of 300 μg/mL AmB in sterile buffer solution (PBS) was prepared and stored at −20 °C. Later, this stock solution was applied in each plate well seeded with both cells lines resulting in final concentrations of 30 μg/mL or 4 μg/mL of AmB depending on the assay. The PKA Pathway Inhibitor (H89 – Calbiochem Merck KGaA, Darmstadt, Germany) was dissolved in anhydrous dimethylsulfoxide (DMSO) to form a stock solution that was 1000 times the required final concentration. The inhibitor stock solution was aliquoted and stored at −20 °C. The stock solution was diluted immediately prior to use and cells were pretreated with 1.0 μM of H89 for 30 min. Cellular viability when cells were exposed to H89 was 93% (Chaves et al., 2009). The concentration of db-cAMP (N6, 2′-O-dibutiriladenosina 3′: 5′-cyclic monophosphate-db-cAMP – Sigma, St. Louis, MO) use solution was 10^{-5} M (Chaves et al., 2008).

Cell culture

Considering the complexity of the kidney, we used in this study two different cell lines from different portions of the renal tissue as a way of improving our experimental model. Cell lines LLC-PK1 (porcine kidney proximal tubular cells – passages 10–20) and MDCK cells (canine distal tubular cells – passages 5–15) were obtained from the Cell Bank at Universidade Federal do Rio de Janeiro (UFRJ). They were cultivated in an RPMI-1640 culture medium (Sigma St. Louis, MO) and supplemented with 10% (v/v) bovine fetal serum (Invitrogen Co Ltd, Carlsbad, CA), 100 IU penicillin/mL, and 100 μg streptomycin/mL (Sigma, St. Louis, MO). We used RPMI-1640 culture medium because it contains all the substances necessary to maintain cell integrity and proliferation. Cells were cultivated in 75 cm^2 flasks (TPP, Sigma Aldrich, St. Louis, MO) and kept at 37 °C with 5% CO_2.

MTT assay

MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay was performed based on the protocol proposed by Mosman (1983). To study the involvement of cAMP in protection against AmB (Cristália Produtos Químicos Farmacêuticos Ltda, Itapira, SP, Brazil) induced cell death, LLC-PK1, and MDCK cells were treated with 10^{-5} M db-cAMP (commercial analogue of cAMP – Sigma Aldrich, St. Louis, MO), followed by AmB (30.0 μg/mL) treatment. The concentration of 30.0 μg/mL of AmB represents the IC 50 value for both cells lines as assessed by dose-dependent curve (data not shown). Cells were seeded (5.0×10^3 cells/well) on 96-well plates (TPP, Sigma Aldrich, St. Louis, MO) and incubated with AmB and/or db-cAMP (Sigma Aldrich, St. Louis, MO) for 24 h at 37 °C, 5% CO_2. Having completed the exposure time, the medium containing the drug was removed, 20 μL of MTT (Sigma Aldrich, St. Louis, MO) solution (5.0 mg/mL) was added, and plates were incubated for one hour at 37 °C, 5% CO_2. The MTT solution was then removed, and 100 μL of DMSO (Sigma Aldrich, St. Louis, MO) was added to each well. The absorbance was read at 570 nm (Thermo Plate model TP-READER, Molecular Devices, Oceanside, CA) and the results were expressed as a percentage of the viability present in treated cells compared with negative control cells. Tests were performed in sextuplicates from three independent experiments.

Flow cytometry assays

A flow cytometry DNA fragmentation assay was employed as a quantitative measure of cell death (Ricardi & Nicoletti, 2006). To study the involvement of cAMP in protection against AmB induced cell death, LLC-PK1 and MDCK cells were seeded in 24 well plates (TPP, Sigma Aldrich, St. Louis, MO) and treated with 10^{-5} M db-cAMP (commercial analogue of cAMP) followed by AmB (4.0 μg/mL) treatment. To study the involvement of PKA signaling pathway in cell protection against AmB, cells were pretreated for 30 min with 1.0 μM H89 (PKA activation inhibitor), followed by AmB (4.0 μg/mL) and 10^{-5} M db-cAMP treatment. Twenty-four hours after treatment, the supernatant of each well was collected and centrifuged. The resulting pellet was combined to cells that were lysed and collected from each corresponding well by the exposure to 300 μL of a hypotonic solution containing 0.5% Triton X-100 and 50 μg/mL propidium iodide (propidium iodide, Invitrogen, Carlsbad, CA). Cell lyses was optimized by homogenization by pippeting the material up and down. Cells were incubated at 4 °C for 1 h and analyzed in a flow cytometer (Guava easyCyte BHT – Millipore, Billerica, MA) for shifts in propidium iodide fluorescence that were indicative of nuclei with hypodiploid DNA content. Assays were performed in triplicates from three independent experiments.

Western blot assay

MDCK and LLC-PK1 cells were grown on 75 cm^2 flasks until 90% confluence was reached. Next, cells were exposed to different substances in accordance with the following groups: negative control (untreated cells); positive control – 10 μM forskolin (Burgos et al., 2004) (Sigma-Aldrich, St. Louis, MO, Sigma®); cells treated with db-cAMP 10^{-5} M; cells treated with AmB (4.0 μg/mL); cells treated with H89 (1.0 μM); cells treated with AmB (4.0 μg/mL) associated with db-cAMP (20 μM); cells pre-treated with H89 (1.0 μM) for 30 min following AmB (4.0 μg/mL) associated with db-cAMP (10^{-5} M) After chemical additions, cells were incubated for three hours at 37 °C, 5% CO_2. Next, cells were collected using trypsin. After centrifugation of samples, the supernatant was discarded and the precipitate was resuspended in 500 μL of lysis buffer (the lysis buffer is a solution composed by Tris HCl 1.0 mM; EDTA 0.5 M; NaCl 5.0 M; DTT; Nonidet P40; protease inhibitor cocktail – Sigma-Aldrich, St. Louis, MO – Sigma®). The homogenate was incubated on ice for 10 min, and next the samples were centrifuged for 2 h at 4380*g* at 4 °C. The supernatant was collected and transferred to a new tube, and proteins were finally quantified using Bradford (1976) assay in a

spectrophotometer (spectrophotometer cuvette digital – Biosystems, Waltham, MA) at 595 nm (Burgos et al., 2004). The sample buffer used to prepare the samples for loading the gel was 5 × Loading Buffer (Fementas – Thermo Fisher Scientific Inc., Waltham, MA). After preparing the samples, they were incubated for 5 min in 95 °C. Next, proteins were loaded in a 10% polyacrilamyde gel. After the electrophoresis, the proteins were electrotransfered onto a nitrocellulose membrane adapting the methodology previously described (Sambrook et al., 1989) and then incubated with the primary antibody (rabbit anti-PRKACG antibody polyclonal (Sigma-Aldrich, St. Louis, MO), with a dilution factor of 1:250 for 12 h at 4 °C. To effectively assess that our target in western blot assay was in fact the active form of PKA, we used this specific antibody that links exclusively to the phosphorilated form of this protein. After incubation, the membranes were extensively washed with TBST buffer (500 mmol/L NaCl, 20 mmol/L Tris-HCL, and 0.4% Tween 20; pH 7.4), followed by the secondary antibody incubation (anti-rabbit IgG-peroxidase, antibody produced in goat, Sigma-Aldrich, St. Louis, MO) for 2 h at 4 °C. The secondary antibody was diluted by a factor of 1:10 000, and the same extensive washes with TBST were performed. Finally the bands were visualized after incubation with Luminol Enhancer Solution (GE Healthcare, Menlo Park, CA) for 1 min, followed by a 15-s exposure to Hyperfilm-ECL (GE Healthcare, Menlo Park, CA). The results were quantified using Quantity One, Bio-Rad, Hercules, CA.

Statistical analysis

All results were analyzed by the ANOVA and the Tukey post-test. The results obtained in Western Blot assays were first analyzed by image analysis software Quantity One, Bio-Rad, Hercules, CA.

Results

Influence of cAMP in cellular viability after treatment with amphotericin B

Both cell lines showed a similar response to AmB, with lower MTT metabolization compared with negative control groups which suggests a decrease in cell viability ($p < 0.05$) (Figure 1 – panels A and B). Groups exposed to db-cAMP from both cell lines had no changes in MTT metabolization in comparison to negative controls. There is a significant increase ($p < 0.05$) in MTT metabolization in the groups submitted to the association of drug and db-cAMP compared with the AmB group in both LLC-PK1 and MDCK cells. This suggests that cell viability increases when cells are exposed to this drug associated to db-cAMP compared with the groups exposed only to AmB. There was no significant difference between cell lines despite the fact that they represent different areas of the renal tissue. Further studies must be done to explain this aspect of the results.

cAMP influence in renal cells lines integrity when submitted to amphotericin B

LLC-PK1 and MDCK cells showed an increased rate of DNA fragmentation when submitted to AmB compared with the negative control groups (Figure 2 – panels A and B). Groups submitted to the association of drug and db-cAMP showed a decrease DNA fragmentation, which suggests a decline in cell death events compared with the groups exposed to AmB only ($p < 0.05$). In order to verify the hypothesis that the observed protective effect would be carried by PKA, we carried out another flow cytometry experiment in which cells were previously exposed to H89, a potent inhibitor of PKA activation (Reber et al., 2012), and then submitted to treatment with the association of drug and db-cAMP. Results showed a significant decrease in DNA

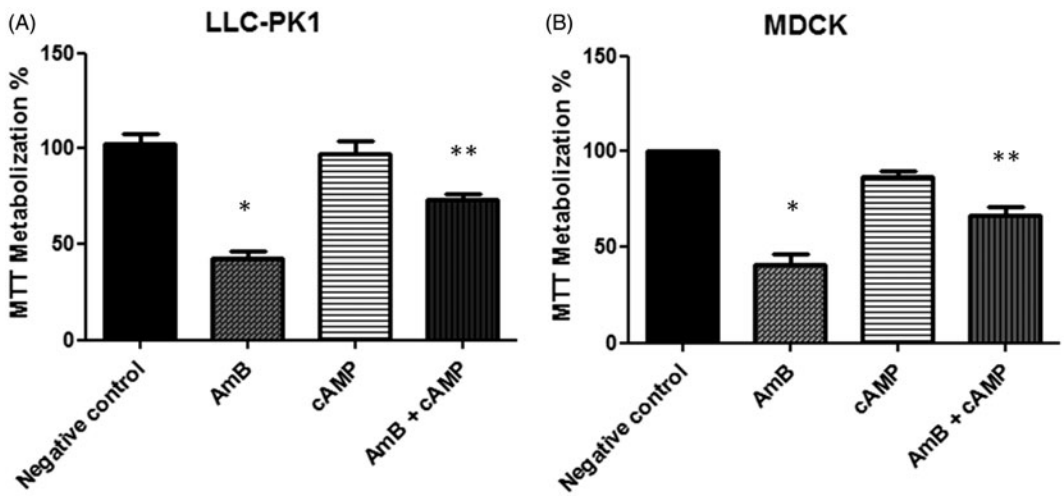

Figure 1. Influence of db-cAMP in cellular viability after AmB treatment. LLC-PK-1 (A) and MDCK (B) cells were seeded in 96-well plates (5.0×10^3 cells/well) and were treated with AmB, db-cAMP (cAMP analog) and AmB + db-cAMP. Cell viability was analyzed by MTT assay. MTT dye reduction was expressed as MTT metabolization %. The absorbance of group treated with AmB and group treated with db-cAMP was compared with the negative control group's (100% MTT metabolization was considered for the negative control group) for both cell lines. The absorbance of group treated with AmB + db-cAMP was compared with the group treated with AmB. Results represent mean ± SD of sextuplicates from three independent experiments. *$p < 0.05$ when compared with the negative control group (untreated cells) and **$p < 0.05$ when compared with AmB.

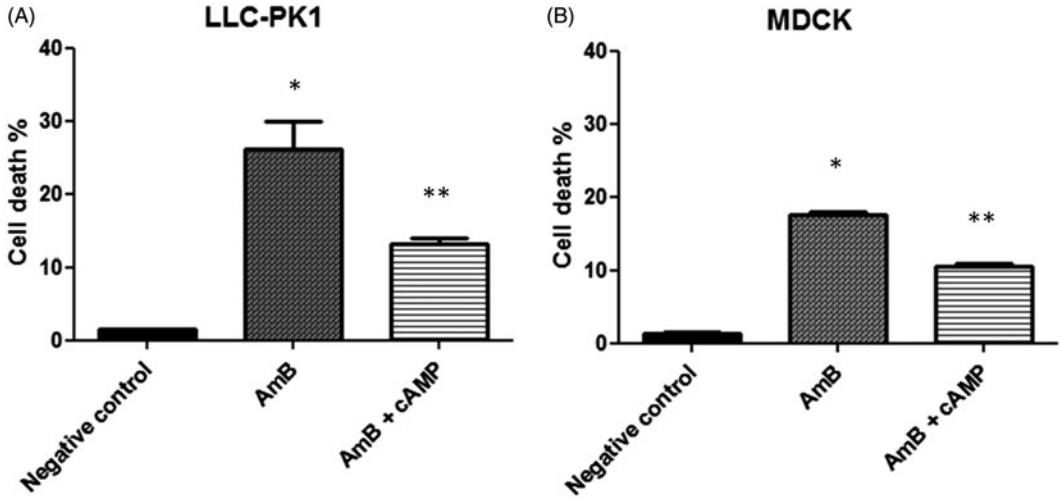

Figure 2. cAMP (db-cAMP) influence in LLC-PK1 (A) and MDCK (B) cells integrity when submitted to AmB. Cells from both cell lines were seeded in 24-well plates (1.0×10^4 cells/well) and were treated with AmB (4.0 μg/mL) and AmB + cAMP in triplicates. Cell state of integrity was evaluated as cell death percentage and was analyzed after staining with PI. A flow cytometry assay was employed as a quantitative measure of DNA fragmentation which suggests cell death. Results are expressed as percentage of events from a total of 5000 events. Results represent mean ± SD of triplicates ($n = 3$) from three independent experiments. * and **mean significantly different from negative control or group treated with AmB ($p < 0.05$).

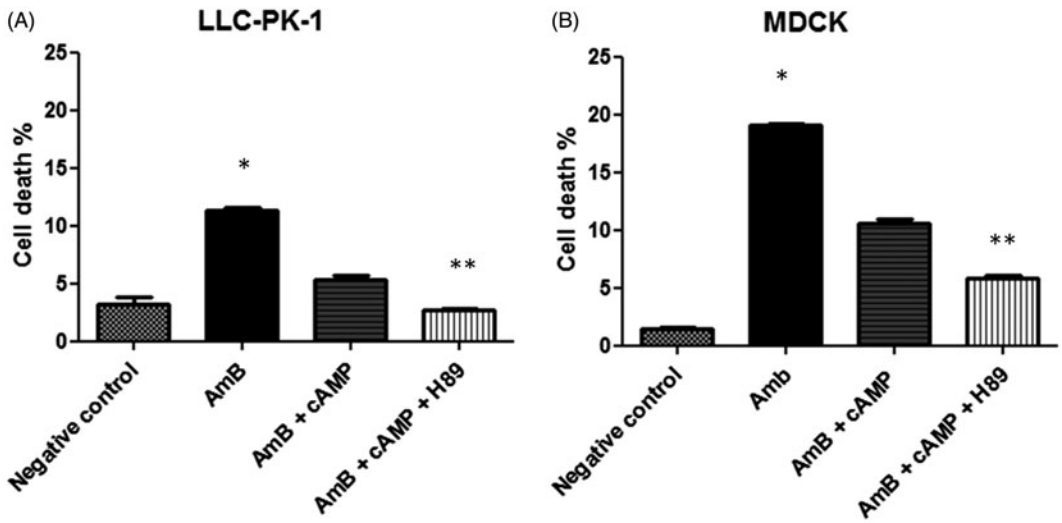

Figure 3. H89 influence in LLC-PK1 (A) and MDCK (B) cells integrity when submitted to AmB associated with cAMP – flow cytometry assay. Cell integrity was expressed as percentage of cell death. Cells were seeded in a 24-well plate (1.0×10^4 cells/well) and were treated with AmB, AmB + cAMP (db-cAMP, a cAMP analog) and AmB + cAMP + H89 (PKA inhibitor) in triplicates. DNA fragmentation was analyzed after staining with PI. The flow cytometry assay was employed as a quantitative measure of DNA fragmentation which suggests cell death. Results are expressed as percentage of events from a total of 5000 events. Results represent mean ± SD of triplicates ($n = 3$) from three independent experiments. * and **mean significantly different from negative control or group treated with AmB + cAMP ($p < 0.05$).

fragmentation when cells from both lines are subjected to the combination of AmB and db-cAMP subsequent to treatment with the inhibitor H89 compared with the group exposed only to the association (AmB and db-cAMP), and this suggests a decrease in cell death events (Figure 3). This suggests that the protective effect of cAMP is independent from PKA activation in both MDCK and LLCPK-1. Similar to the results from the MTT assay, there was no difference between the responses from the cell lines despite the fact that they represent different areas of the renal tissue. Further studies must be conducted to explain why this is so.

Western blot assay

Results in Figure 4 showed the involvement of the cAMP signaling pathway in the nephrotoxicity of AmB. Positive control (Forskolin), cAMP, and the association between AmB and db-cAMP induced a significant activation of PKA in LLC-PK1 and MDCK cell lines (Figure 4 – panels A and B). Cells exposed to AmB did not show significant increase in PKA activation compared with negative control in both cell lines. When cells were pretreated with H89 and then exposed to the association of AmB and db-cAMP, a significant

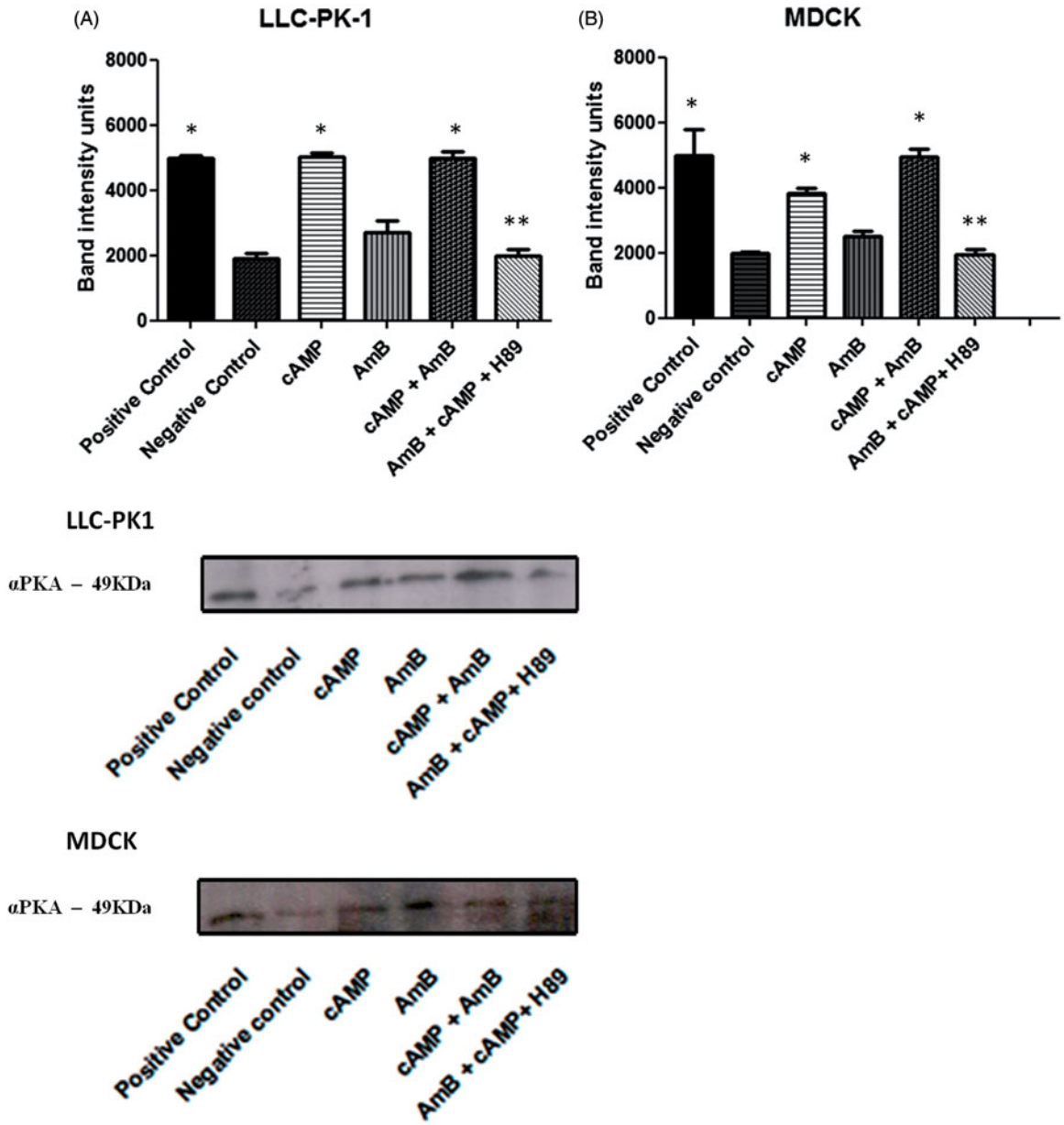

Figure 4. H89 influence in PKA activation in LLC-PK1 and MDCK cells when submitted to amphotericin B associated with cAMP – Western blot assay. Positive control: Forskolin (10 μM); negative control: untreated cells; cAMP (db-cAMP); AmB (4.0 μg/mL). *$p < 0.05$ for values significantly different from the negative control groups. **$p < 0.05$ for values different from those of the groups exposed to the association between AmB and db-cAMP. Image analysis was done by software Quantity One, Bio-Rad, Hercules, CA, and later statistical analysis was performed by the ANOVA and the Tukey post-test. The analyses were performed in triplicate ($n = 3$).

decrease in band intensity could be observed when compared with the group treated with AmB and db-cAMP (Figure 4 – panels A and B).

Discussion

The present study demonstrates that db-cAMP may protect renal cell lines against AmB toxicity in a PKA-independent manner. This was shown when two different cell lines, one of proximal tubular epithelial origin (LLC-PK-1) and the other composed of collecting duct cells (MDCK) were exposed to db-cAMP in association with AmB treatment. An increase in cell survival could be observed when comparing groups exposed to the association between drug and db-cAMP and the groups exposed to AmB only as shown by MTT and flow cytometry assays. Flow cytometry assay results also showed that this cytoprotective effect occurred independently from PKA activation, in both cell lines, as H89 exposure, prior to the association between drug and db-cAMP incubation, did not alter the protective effect. Western Blot assays, performed in both cell lines, confirmed that there was no activation of PKA in groups exposed to H89. Db-cAMP has the capacity to trigger other proteins that are inducible by cAMP besides PKA (Spina et al., 2012).

Nowadays, many drugs used in therapeutics demonstrate severe nephrotoxic potential and drug-induced acute kidney injury (AKI) have been implicated in 8–60% of all cases of in-hospital AKI. As such, AKI is recognized as a source of

significant morbidity and mortality (Khalili et al., 2013). This makes designing strategies that can effectively reduce such effects imperative (Mishima et al., 2006).

A study conducted by Stokman showed that renal epithelial cells subjected to ischemia, followed by reperfusion (which commonly contributes to renal failure), leading to a loss of integrity of cell–cell junctions, were capable of maintaining their integrity when submitted to incubation with another molecule, 8-PCPT-2′-O-Me-cAMP, in a PKA-independent manner, since that particular cAMP analog lacks PKA activation capacity (Stokman et al., 2011).

The intrarenal administration of 8-PCPT-2′-O-Me-cAMP *in vivo* led to the same effect when mice underwent renal injury caused by ischemia followed by reperfusion. This study demonstrated the importance of cell–cell interactions preservation, and cAMP cell adhesion-dependent signaling pathways to resist against renal damage in a new way, involving the Rap protein family.

Other studies have also proven that cAMP plays a key protective role against kidney injury as an adverse effect of cisplatin use (Arany et al., 2008; Li et al., 2010; Mishima et al., 2006). Also, there is evidence that the use of 8-PCPT-2′-O-Me-cAMP in mice kidney proximal tubule cells leads to the preservation of the integrity of those cells when exposed to cisplatin, a highly nephrotoxic drug, by preventing cell detachment and apoptosis (Qin et al., 2012). These findings are in agreement with the present study, as MDCK and LLC-PK-1 cells resisted cell death due to exposure to AmB when submitted to the association between db-cAMP and AmB.

AmB is still considered the first-line treatment for cryptococcosis and histoplasmosis (Johnson et al., 2002; Moen et al., 2009; Saag et al., 2000). There are also reports of its use in resistance to azoles, whether alone or in combination (Chandrasekar, 2011), even with its high nephrotoxicity. It has been proposed that AmB toxicity is due to its capacity to provoke a loss of osmolarity in proximal tubular cells, afferent arteriolar vasoconstriction followed by reperfusion, and an elevated pro-inflammatory cytokine response, which all result in cell death (Louis et al., 2013). The present study suggests that db-cAMP may prevent cell death in LLC-PK-1 and MDCK cell lines due to AmB exposure. Further studies are necessary to assess the precise mechanism involved in this process. It should be investigated if the effect initiated by cAMP is carried out by ion channels or if other proteins are responsible for it, as Epac, for example. When cAMP activates Epac, it functions as an exchange factor for the small GTPase Rap1, mediating replacement of GDP for GTP, consequently leading to activation of Rap1. Among the processes known to be influenced by Rap1 are integrin-mediated adhesion to the extracellular matrix and the preservation of cell–cell contacts (Hogan et al., 2004). Cyclic adenosime monophosphate also regulates electrogenic absorption of Na+ by the amiloride-sensitive epithelial Na+ channel, which is essential for the maintenance of sodium equilibrium in renal tissue (Honegger et al., 2006). It is important to know if the protective effect is a result of ion channels or Epac activation. If Epac is involved, then it would be important to assess if it is the ability of this protein in maintaining cell–cell interactions and cell adhesion the reason why there is protection of renal cells against amphotericin B nephrotoxicity.

Conclusion

It can be concluded that cAMP (db-cAMP) could protect renal cells from death due to AmB exposure. This is the first time that this effect is shown when renal cells are exposed to the nephrotoxic potential of AmB.

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Declaration of interest

The authors report that they have no conflicts of interest. UFMG (Universidade Federal de Minas Gerais), UFOP (Universidade Federal de Ouro Preto), FAPEMIG (Fundação de Amparo e Pesquisa de Minas Gerais Log Number: APQ-00596-08), CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior), and CNPQ (Conselho Nacional de Pesquisa) supported this paper.

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