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The involvement of protein kinase A in the immune response of *Galleria mellonella* larvae to bacteria

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The role of protein kinase A (PKA) in the humoral immune response of the greater wax moth *Galleria mellonella* larvae to live Gram-positive bacteria *Micrococcus lysodeikticus* and Gram-negative bacteria *Escherichia coli* was investigated. The immune challenge of larvae with both kinds of bacteria caused an increase in fat body PKA activity depending on the injected bacteria. Gram-positive *M. lysodeikticus* was a much better inducer of the enzyme activity than Gram-negative *E. coli*. The PKA activity was increased about 2.5-fold and 1.5-fold, after *M. lysodeikticus* and *E. coli* injection, respectively. The *in vivo* inhibition of the enzyme activity by a cell permeable selective PKA inhibitor, Rp-8-Br-cAMPS, was correlated with considerable changes of fat body lysozyme content and hemolymph antimicrobial activity in bacteria-challenged insects. The kinetics of changes were different and dependent on the bacteria used for the immune challenge of *G. mellonella* larvae.

Keywords: *Galleria mellonella*, protein kinase A, lysozyme, antibacterial activity, Rp-8-Br-cAMPS

INTRODUCTION

The characteristic feature of invertebrate immunity is the lack of an adaptive immune response. In insects, pathogen recognition leads to activation of humoral and cellular innate defence mechanisms. The cellular response comprises phagocytosis, encapsulation and nodulation of non-self bodies. In the humoral response, a crucial role is played by antimicrobial peptides, synthesized in the fat body (a functional analog of mammalian liver) and hemocytes after non-self recognition and then released into the hemolymph. Literature data indicated the involvement of cAMP-dependent protein kinase (protein kinase A, PKA) in the regulation of the immune response in insects. Brooks and Dunphy (2005) demonstrated that active PKA limited the greater wax moth *Galleria mellonella* hemocyte response against *Xenorhabdus nematophila* and *Bacillus subtilis in vitro* and *in vivo*. PKA inhibition by a selective inhibitor, Rp-8-Br-cAMPS (Rp-isomer of 8-bromoadenosine 3′,5′-cyclic monophosphorothioate), increased the number of hemocytes with adherent bacterial cells and enhanced phagocytosis of bacteria *in vitro* as well as the removal of bacteria from hemolymph *in vivo* (Brooks & Dunphy, 2005). In contrast, an elevation of cellular cAMP concentration led to an impaired response of *G. mellonella* hemocytes to both kinds of bacteria (Brooks & Dunphy, 2005; Marin *et al.*, 2005). Moreover, the inhibition of PKA by another inhibitor, H89 (*N*-[2-(*p*-bromocinnamylamino)-ethyl]-5-iso-quinolinesulfonamide), increased the adhesion of *G. mellonella* granulocytes to glass slides, indicating PKA involvement in the regulation of hemocyte adhesion properties (Zakarian *et al.*, 2003).

PKA was implicated in the regulation of antimicrobial peptide synthesis in insects. It was suggested that PKA is one of the necessary factors for

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Abbreviations: cfu, colony forming units; EWL, egg white lysozyme; H89, *N*-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide; LPS, lipopolysaccharide; PG, peptidoglycan; PKA, protein kinase A; PMSF, phenylmethylsulfonyl fluoride; PTU, phenylthiourea; Rp-8-Br-cAMPS, Rp-isomer of 8-bromoadenosine 3′,5′-cyclic monophosphorothioate.

the activation of cecropin B genes in isolated *Bombyx mori* hemocytes (Taniai *et al.*, 1996). The expression of cecropin B gene was triggered by dibutyryl-cAMP, a cell-permeable cAMP analog (Shimabukuro *et al.*, 1996). In addition, the expression of that gene induced by LPS or lipid A was inhibited by H89, a PKA inhibitor (Shimabukuro *et al.*, 1996; Taniai *et al.*, 1996).

In *Drosophila melanogaster* at least two signalling pathways, Toll and Imd, are involved in the regulation of antimicrobial peptide gene expression (Leclerc & Reichhart, 2004; Iwanaga & Lee, 2005; Tanji & Ip, 2005; Pinheiro & Ellar, 2006; Wang & Ligoxygakis, 2006). The activation of Toll and Imd pathways leads to the release of NFκB/Rel family transcription factors Dif, Dorsal, Relish. Nuclear transport and activation of *D. melanogaster* factor Dorsal was dependent on phosphorylation by PKA (Norris & Manley, 1992) and it was shown that the phosphorylation of Dorsal by PKA at Ser312 facilitated an interaction of Dorsal molecule with importin (Briggs *et al.*, 1998).

In our recent paper, we demonstrated PKA activity in the fat body of *G. mellonella* larvae (Cytryńska *et al.*, 2006). The enzyme activity was considerably increased after the immune challenge of animals with *Escherichia coli* LPS. The inhibition of PKA by H89 or Rp-8-Br-cAMPS *in vivo* was correlated with a lower level of antimicrobial activity in the hemolymph of immune-challenged insects. A considerable decrease in the content of lysozyme, which plays an important role in the lepidopteran insect immune response, was also detected in the fat body of inhibitor pre-treated LPS-challenged larvae. Our results demonstrated the involvement of PKA in *G. mellonella* humoral immune response to LPS, a cell wall component of Gram-negative bacteria.

In this paper, we investigated a possible role of PKA in the humoral immune response of *G. mellonella* larvae to live Gram-negative bacteria *E. coli* and Gram-positive bacteria *Micrococcus lysodeikticus*. We used a cell permeable, selective PKA inhibitor, Rp-8-Br-cAMPS, and examined the effect of PKA inhibition *in vivo* on hemolymph antimicrobial activity and lysozyme content in the fat body of bacteria-challenged insects.

MATERIALS AND METHODS

Insect culture, immune challenge and hemolymph collection. Larvae of the greater wax moth *Galleria mellonella* (*Lepidoptera: Pyralidae*) were reared on a natural diet — honeybee nest debris at 30°C in the dark. Last instar larvae (250–300 mg in weight) were used throughout the study.

For PKA activity inhibition *in vivo*, the larvae were injected with 1.5 nmol of Rp-8-Br-cAMPS (Sigma) in 3 μl of apyrogenic water (the approximate concentration of the inhibitor in larval hemolymph — 20 μM). Control animals were injected with the same volume of apyrogenic water. In some experiments, 15 min after inhibitor administration, the larvae were immune-challenged by injection of 3 μl of apyrogenic water containing live *E. coli* D31 (10^5 cfu) or *M. lysodeikticus* (5×10^4 cfu). After the treatment, the larvae were kept at 30°C in the dark on sterile Petri dishes and the hemolymph was collected after the time indicated in the text.

The hemolymph collection was performed into sterile and chilled Eppendorf tubes containing a few crystals of phenylthiourea to prevent melanisation, as described earlier (Cytryńska *et al.*, 2006). The hemocyte-free hemolymph was obtained by centrifugation at $200\times g$ for 5 min to pellet hemocytes and the supernatant was subsequently centrifuged at $20\,000\times g$ for 15 min at 4°C to pellet cell debris. The cell-free hemolymph was used immediately for testing antibacterial activity.

Antibacterial activity assay. For antibacterial activity tests, the LPS defective, streptomycin and ampicillin resistant mutant of *E. coli* K12, strain D31 was used (Boman *et al.*, 1974). The antibacterial activity in the hemolymph was detected by a well-diffusion method using solid agar plates containing viable *E. coli* cells (Hoffmann *et al.*, 1981) and hen egg white lysozyme (EWL) at a concentration of 2.0 $mg\times ml^{-1}$ to improve the method sensitivity (Chalk & Suliaman, 1998; Cytryńska *et al.*, 2001). Each well on the Petri dish was filled with 4 μl of hemolymph diluted four times with sterile water. After incubation of the agar plates at 37°C for 24 h the diameters of *E. coli* D31 growth inhibition zones were measured and the level of antimicrobial activity was calculated using the algorithm described by Hultmark *et al*. (1982). For evaluation of antibacterial activity, synthetic cecropin B (Sigma) was used as a standard.

Preparation of hemolymph extracts. Acidic/methanolic extracts of hemocyte-free hemolymph were obtained by the method adapted from Schoofs *et al*. (1990). The hemolymph was diluted ten times with the extraction solution consisting of methanol/glacial acetic acid/water (90:1:9, by vol.) and mixed thoroughly. Precipitated proteins were pelleted by centrifugation at $20\,000\times g$ for 30 min at 4°C. The obtained supernatant, containing peptides and proteins of molecular mass below 30 kDa, was collected, freeze-dried and the pellet was stored at –20°C until needed. For SDS/PAGE, the pellet was dissolved in an appropriate volume of sample buffer according to Schägger and von Jagow (1987).

Isolation of fat bodies and preparation of cell-free extracts. For fat body isolation, the larvae were anaesthesized by submerging in ice-cold apyrogenic water and then surface disinfected with 70% ethanol. The fat bodies were dissected under sterile ice-cold physiological saline (172 mM KCl, 68 mM NaCl, 5 mM $NaHCO_3$, pH 6.1, osmolarity 420 mOsm) (Vilcinskas & Matha, 1997). After dissection, the fat body was transferred into a sterile, chilled Eppendorf tube containing 1 ml of physiological saline. Then the solution was removed and the fat body was frozen in liquid nitrogen.

Cell-free extracts of fat bodies were prepared in ice-cold PKA buffer (50 mM Tris/HCl, pH 7.4, 10 mM β-glycerophosphate, 2.5 mM sodium pyrophosphate, 0.5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 6 mM β-mercaptoethanol), as described earlier (Cytryńska *et al.,* 2006). The obtained extracts were centrifuged at $20000 \times g$ for 15 min at 4°C, the supernatants were collected and were used immediately for PKA activity assay. For immunoblotting, an appropriate volume of Laemmli sample buffer (Laemmli, 1970) was added and the samples were stored at –20°C.

Protein kinase A activity assay. PKA activity was measured by a non-radioactive method using PepTag® Assay (Promega), as described (Cytryńska *et al.,* 2006). Briefly, the reaction mixture in the volume of 25 μl contained: 20 mM Tris/HCl, pH 7.4, 10 mM $MgCl_2$, 1 mM ATP, 1 μM cAMP, 2.5 mM sodium pyrophosphate, 2.5 mM β-glycerophosphate, 2 μg of PepTag® A1 Peptide (specific PKA substrate) and 20 μg of fat body total proteins. Each reaction was performed at room temperature for 30 min and then stopped by heating the incubation mixture at 95°C for 10 min. PepTag® A1 Peptide fluorescence was visualized after 20 min of horizontal electrophoresis in 0.8% agarose by using a UV transilluminator.

Immunoblotting. Samples of fat body extracts (80 μg of total protein) were subjected to 13.8% glycine SDS/PAGE and electroblotted onto Immobilon membranes (Millipore) for 90 min at 350 mA. For lysozyme identification, the membranes were probed with polyclonal antibodies (1 : 1 000) to *G. mellonella* lysozyme, a generous gift of Prof. I. H. Lee (Department of Life Science, Hoseo University, South Korea). As second antibodies, alkaline phosphatase-conjugated goat anti-rabbit IgGs (1 : 30 000) were used and immunoreactive bands were visualized by incubation with *p*-Nitroblue Tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate.

Other methods. The protein concentration was estimated by the Bradford method using bovine serum albumin (BSA) as a standard (Bradford, 1976).

Polyacrylamide gel electrophoresis of proteins was performed by 13.8% glycine SDS/PAGE according to Laemmli (1970). Low molecular mass proteins and peptides were resolved by 16.5% tricine SDS/PAGE according to Schägger and von Jagow (1987).

The densitometric analysis of bands was performed using Quantity One computer imaging system (BioRad, Hercules, CA, USA). All data are presented as means ±S.D. for at least three experiments.

In order to compare two means, statistical analysis was performed by Student's *t*-test. Significance was established at $P<0.001$ (Figs. 1, 2, 3) or $P<0.01$ (Fig. 4).

RESULTS

PKA activity in the fat body of bacteria-challenged *G. mellonella* larvae

In the present work, we investigated the level of PKA activity in the fat body of bacteria-challenged insects. In general, bacterial challenge caused an increase in PKA activity but the kinetics and the level of the enzyme activity differed considerably depending on the injected bacteria. In the larvae challenged with Gram-positive bacteria *M. lysodeikticus*, a considerable increase in the enzyme activity was noticed when compared to the water-treated larvae (Fig. 1). About a 2.5-fold increase in PKA activity was detected as early as 15 min after the challenge and a high level of enzyme activity was sustained until 24 h after injection of bacteria (Fig. 1).

When *G. mellonella* larvae were immune-challenged with Gram-negative bacteria *E. coli*, a different kinetics of fat body PKA activity was observed (Fig. 1). In general, the enzyme activity level in *E. coli*-challenged larvae was lower in comparison to the level measured in *M. lysodeikticus*-injected insects. In most time points after the challenge, PKA activity oscillated around the naïve larvae level, however, starting from about 15 min after the treatment, it was increased when compared to water-treated animals. A slight, nevertheless significant ($P<0.001$) increase in the enzyme activity was detected 45 min after bacteria injection. A more considerable increase in PKA activity, 1.5-fold and 1.6–1.8-fold, respectively, was detected 2 and then 6–24 h after the challenge, in comparison to the measurements in water-treated larvae. However, a transient decrease by 29.6% of PKA activity was detected 4 h after treatment with *E. coli* (Fig. 1).

When *G. mellonella* larvae, before bacteria injection, were pre-treated with PKA inhibitor, Rp-8-Br-cAMPS, a fall in PKA activity was noticed

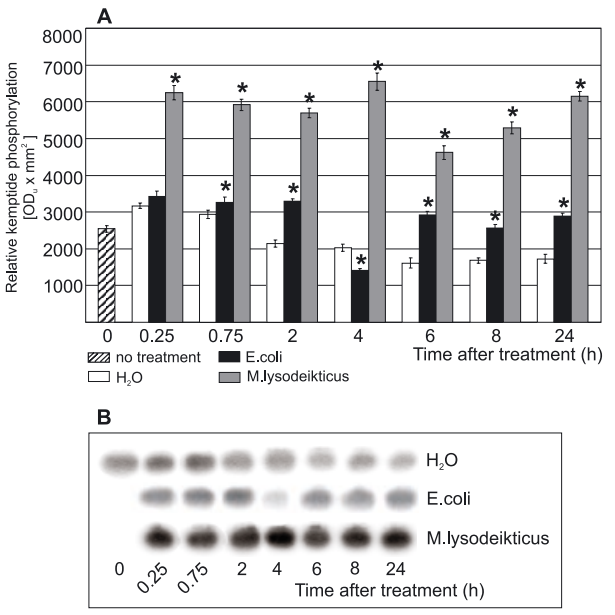

Figure 1. PKA activity in the fat body of bacteria-challenged *G. mellonella* larvae.
The larvae (five individuals per each group) were injected with water, *M. lysodeikticus* or *E. coli* suspension as described in Materials and Methods. The fat bodies were isolated at the indicated time points, cell-free extracts were prepared and PKA activity was measured. Diagram (A) presents the densitometric analysis of bands containing phosphorylated kemptide and photo (B) shows corresponding fragments of the agarose gels. The first hatched bar represents PKA activity in the fat body of non-treated larvae. *Statistically significant differences ($P<0.001$) from samples after H_2O treatment at respective time points.

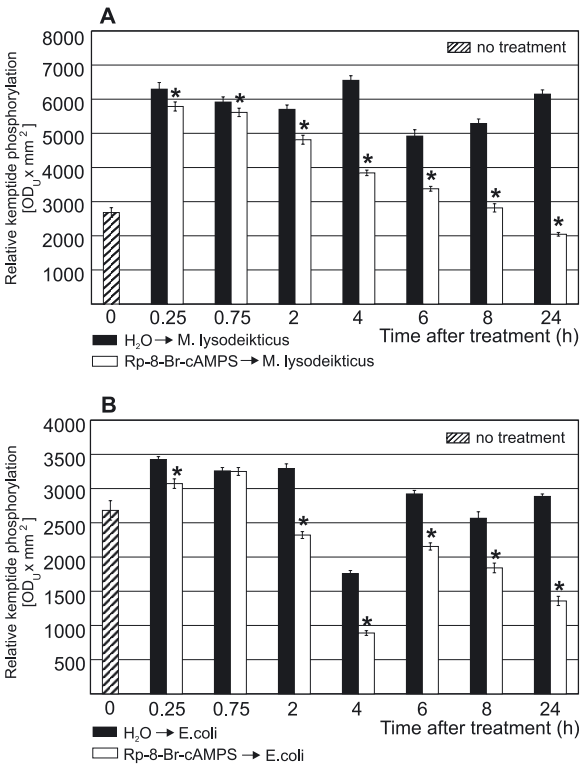

Figure 2. PKA activity in the fat body of *G. mellonella* larvae challenged with *M. lysodeikticus* (A) and *E. coli* (B) pre-treated with PKA inhibitor.
The larvae (five individuals per each group) were injected with Rp-8-Br-cAMPS or water and then with suspension of *M. lysodeikticus* or *E. coli*. The fat bodies were isolated at the indicated time points and PKA activity was measured as described in Materials and Methods. The first hatched bars represent PKA activity in fat body of non-treated larvae. *Statistically significant differences ($P<0.001$) Rp-8-Br-cAMPS *versus* no Rp-8-Br-cAMPS at respective time points.

(Fig. 2). Fifteen minutes after the challenge with *M. lysodeikticus*, the PKA activity was decreased by 7.4%. A more evident impairment of PKA activity, by 27.5–67.3%, was detected 4–24 h after *M. lysodeikticus* injection (Fig. 2A). When PKA inhibitor was used before *E. coli* injection, 15 min after the challenge PKA activity in the fat body was lowered by 10.6% and then, 2–24 h after the challenge, by 25–52.3% (Fig. 2B).

Lysozyme content in the fat body of bacteria-challenged larvae, pre-treated with PKA inhibitor

Literature data indicate that lysozyme plays an important role in insect innate immunity (Powning & Davidson, 1973; Chadwick & Aston, 1991; Hultmark, 1996). The protein is synthesized mainly in the fat body and hemocytes and released into the hemolymph. The constitutive, low level of lysozyme is present in lepidopteran insect hemolymph, however, non-self recognition leads to an increase in the hemolymph lysozyme content and activity (Sun *et al.*, 1991; Morishima *et al.*, 1995; Chung & Ourth, 2000; Lavine *et al.*, 2005). Recently, we demonstrated that PKA activity inhibition was correlated with a considerable decrease in lysozyme content in fat body of LPS-challenged larvae (Cytryńska *et al.*, 2006).

In the present study, we investigated whether similar correlation occurred between PKA activity inhibition and lysozyme content in the fat body of *M. lysodeikticus*- and *E. coli*-challenged larvae (Fig. 3). In the fat body of bacteria-challenged larvae, lysozyme was detected 4 and 6 h after treatment with *M. lysodeikticus* and *E. coli*, respectively, although at a relatively low level (Fig. 3). Then the lysozyme content gradually increased reaching the highest level 24 h after the challenge (Fig. 3A, B). When bacteria-injected insects were pre-treated with PKA inhibitor, lysozyme in the fat body was detected earlier than in water pre-treated insects, which means 2 h after injection of bacteria (Fig. 3A, B). In Rp-8-Br-cAMPS pre-treated *M. lysodeikticus*-challenged larvae, 2 h after the challenge, a high level of the protein was detected (Fig. 3A). The densitometric analysis revealed that it reached 82.4% of the lysozyme content measured in the fat body of water pre-treated lar-

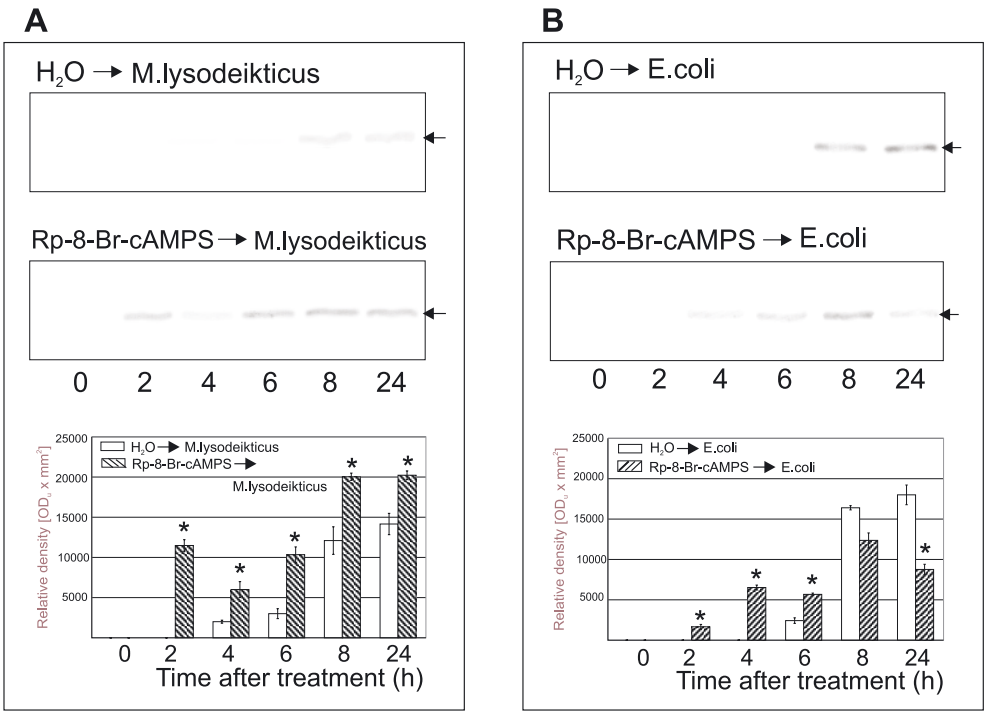

Figure 3. Detection of lysozyme in the fat body of *G. mellonella* larvae challenged with *M. lysodeikticus* (A) and *E. coli* (B) pre-treated with PKA inhibitor.
The larvae (five individuals per each group) were injected with Rp-8-Br-cAMPS or water and then with suspension of *M. lysodeikticus* or *E. coli*. The fat bodies were isolated at the indicated time points and prepared for immunoblotting. The samples were resolved by glycine SDS/PAGE and transferred onto Immobilon membranes. The membranes were probed with antibodies against *G. mellonella* lysozyme. The lysozyme band is marked by an arrow. The diagrams represent the densitometric analysis of bands containing lysozyme. *Statistically significant differences ($P<0.001$) Rp-8-Br-cAMPS *versus* no Rp-8-Br-cAMPS at respective time points.

vae 24 h after challenge. A further increase in lysozyme content, by 4-, 1.6-, and 1.5-fold, respectively, was noticed 6, 8 and 24 h after injection of bacteria. However, 4 h after the challenge in Rp-8-Br-cAMPS pre-treated animals, a transient decrease in lysozyme content was noticed (Fig. 3A).

Different results were obtained when lysozyme content was examined in *E. coli*-challenged larvae pre-treated with PKA inhibitor (Fig. 3B). Starting from about 8 h after *E. coli* injection, the lysozyme content in the fat body of these animals was lowered and 24 h after the challenge it reached 50% of that measured in water pre-treated larvae (Fig. 3B). The results obtained for *E. coli*-challenged larvae resembled, to some extent, those presented for LPS-injected insects pre-treated with PKA inhibitor, where a diminishing in fat body lysozyme content was also detected (Cytryńska *et al.*, 2006).

Antibacterial activity in the hemolymph of bacteria-challenged larvae, pre-treated with PKA inhibitor

It is well documented that bacteria infection induces the synthesis of antimicrobial peptides in the insect fat body which are secreted into the hemolymph of challenged larvae (Dickinson *et al.*, 1988; Yamakawa & Tanaka, 1999; Lowenberger, 2001; Mak *et al.*, 2001; Hultmark, 2003; Royet *et al.*, 2005). We investigated the level of antibacterial activity in the hemolymph of bacteria-challenged larvae pre-treated with PKA inhibitor.

Immune challenge of *G. mellonella* larvae with *M. lysodeikticus* or *E. coli* caused the appearance of antibacterial activity in the hemolymph (Fig. 4). The activity was detected as early as 2 h after the challenge and it gradually increased reaching, 24 h after injection of bacteria, the level equivalent to cecropin B activity of 6 μM and 4.5 μM, for *M. lysodeikticus*- and *E. coli*-challenged larvae, respectively (Fig. 4A, B). The induction of antibacterial activity was correlated with the appearance in the hemolymph of additional peptides of molecular mass below 6.5 kDa (Fig. 4C).

When *M. lysodeikticus*-challenged larvae were pre-treated with PKA inhibitor, the antibacterial activity level measured 3–4 h after the challenge was diminished by 33.3% and 28.6%, respectively, in comparison to water pre-treated animals. In contrast, an increase in antibacterial activity, reaching 1.4-fold 24 h after treatment, was detected 8–24 h after the challenge (Fig. 4A).

Pre-treatment of *G. mellonella* larvae with Rp-8-Br-cAMPS before *E. coli* injection resulted in a lower level of hemolymph antibacterial activity than

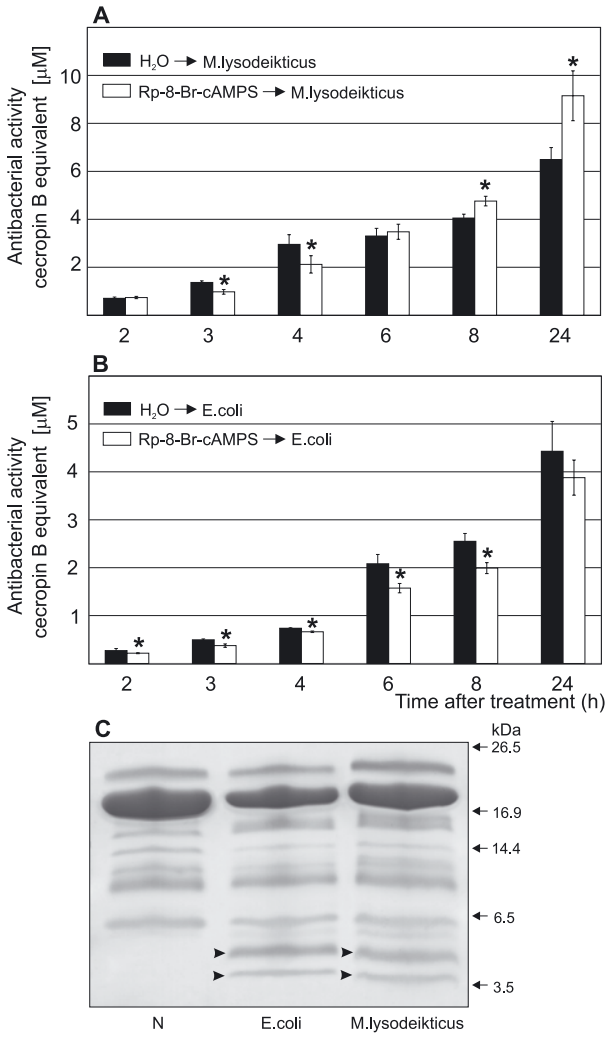

Figure 4. Antibacterial activity in the hemolymph of *G. mellonella* larvae challenged with *M. lysodeikticus* (A) and *E. coli* (B) pre-treated with PKA inhibitor.
The larvae (ten individuals per each group) were injected with Rp-8-Br-cAMPS or water and then with suspension of *M. lysodeikticus* or *E. coli*. The hemolymph was collected at the indicated time points and used for antimicrobial activity assay as described in Materials and Methods. The photo (C) presents tricine SDS/PAGE analysis of extracts prepared from hemolymph obtained 24 h after bacteria injection and from naive larvae hemolymph (N). Additional peptide bands present in hemolymph of bacteria-challenged insects are marked by arrowheads. *Statistically significant differences ($P<0.01$) Rp-8-Br-cAMPS *versus* no Rp-8-Br-cAMPS at respective time points.

in water pre-treated animals (Fig. 4B). In particular, 6 and 8 h after the treatment it reached 74.1% and 78%, respectively, of that measured in the hemolymph of water pre-treated insects (Fig. 4B).

DISCUSSION

We showed recently that the immune challenge of *G. mellonella* larvae with *E. coli* LPS led to a considerable, about 2-fold, increase in fat body PKA activity (Cytryńska *et al.*, 2006). Here we demonstrated that infection of *G. mellonella* larvae with live Gram-positive and Gram-negative bacteria influenced the level of PKA activity depending on the bacteria used for immune challenge. After bacterial treatment, the increased level of PKA activity was sustained for 24 h, similarly to the results obtained for LPS-challenged insects (Cytryńska *et al.*, 2006). However, Gram-positive *M. lysodeikticus* appeared to be a much better inducer of PKA activity than Gram-negative *E. coli*. Although *E. coli* cells (containing LPS) were used for *G. mellonella* challenge in a 2-fold higher amount than *M. lysodeikticus*, PKA activity was induced to a much lower level. It is known that the bacterial cell wall composition is an important determinant of insect immune response induction. Studies performed on *D. melanogaster* indicated that especially the type of peptidoglycan (PG) molecules determined the activation of certain signalling pathways involved in innate immunity mechanisms (Leulier *et al.*, 2003; Kurata, 2004; Kurata *et al.*, 2006). Most Gram-positive bacteria, whose cell walls contain a thick layer of lysine type PG, activate the Toll pathway. The Gram-negative bacteria, containing cell walls composed of a thin layer of diaminopimelic type PG covered by LPS, activate the Imd signalling pathway. The infection of *D. melanogaster* with different classes of bacteria induces preferential synthesis of different repertoires of defence peptides in the fat body (Leclerc & Reichhart, 2004; Tanji & Ip, 2005; Pinheiro & Ellar, 2006; Wang & Ligoxygakis, 2006). Although signalling pathways activated in *G. mellonella* larvae after bacterial infection are not known at present, it was demonstrated that PKA (Cytryńska *et al.*, 2006) and JNK MAP kinase (Wojda *et al.*, 2004) are engaged in the humoral immune response in LPS-challenged *G. mellonella* larvae. The differences in the level and the kinetics of PKA activation after *G. mellonella* challenge with Gram-positive and Gram-negative bacteria indicate the functioning of different signalling pathways responding to both classes of bacteria in which PKA plays a regulatory role.

The use of a cell permeable selective PKA inhibitor, Rp-8-Br-cAMPS, before injection of bacteria, led to a decrease in fat body PKA activity in *M. lysodeikticus*- as well as *E. coli*-challenged larvae. The kinetics of PKA activity changes in Rp-8-Br-cAMPS pre-treated animals might suggest that the enzyme inhibition detected shortly after the challenge evoked further changes in cellular as well as in the whole organism metabolism, which in turn influenced the PKA activity later after the challenge.

The inhibition of PKA activity considerably coincided with changes in the fat body lysozyme content. In the fat body of larvae pre-treated with

PKA inhibitor, lysozyme appeared at least 2 h earlier than in the water pre-treated ones. This seems to indicate that active PKA may to some extent regulate the process of lysozyme synthesis and/or promote a release of this protein from the fat body to the hemolymph in bacteria-challenged larvae. Our data might suggest that PKA, at least within the first hours after the challenge, is involved in negative regulation of lysozyme content in the fat body of bacteria-injected *G. mellonella* larvae. It is consistent with the results of studies on *G. mellonella* cellular immune response, where active PKA limited hemocyte reactions against *X. nematophila* and *B. subtilis* and inhibition of the enzyme enhanced phagocytosis of bacteria and increased adhesion properties of granulocytes (Zakarian *et al.,* 2003; Brooks & Dunphy, 2005; Marin *et al.,* 2005).

Interestingly, changes in fat body lysozyme content under the conditions of PKA inhibition were dependent on the bacteria used for the immune challenge. A considerable increase in the lysozyme content was detected in the fat body of *M. lysodeikticus*-challenged insects. After *E. coli* injection, a decrease in the protein level was observed, resembling the results obtained for LPS-challenged larvae (Cytryńska *et al.,* 2006). This probably reflects differences in immune reaction of *G. mellonella* larvae to both bacteria and also indicates PKA participation in the modulation of these processes.

PKA inhibition also influenced the hemolymph antibacterial activity. The kinetics of changes in the antibacterial activity level in Rp-8-Br-cAMPS pre-treated animals was dependent on the injected bacteria. However, the temporary decrease in the studied activity detected in hemolymph of larvae challenged with both bacteria could suggest the enzyme participation in the regulation of antimicrobial peptides synthesis and/or their release from fat body cells. So far, the engagement of PKA in the regulation of the cecropin B gene in *B. mori* hemocytes has been described (Taniai *et al.,* 1996; Shimabukuro *et al.,* 1996). The differences in antibacterial activity level in hemolymph of *M. lysodeikticus*- and *E. coli*-challenged larvae could be connected with a higher and a lower content of lysozyme released from the fat body, respectively.

It was shown that the low constitutive level of lysozyme present in the hemolymph of lepidopteran insect non-immune larvae plays a significant role in peptidoglycan digestion. Soluble PG fragments released by lysozyme from bacterial cell walls can act as signalling molecules for activation of antimicrobial peptide synthesis, including lysozyme (Dunn *et al.,* 1985; Kanost *et al.,* 1988). Concerning the differences in PG content in cell walls of Gram-positive and Gram-negative bacteria, its accessibility for and susceptibility to lysozyme, one can speculate that considerably more soluble PG fragments might be released from *M. lysodeikticus* than from *E. coli* cell walls. Inhibition of PKA activity together with the presence of different type and amount of PG in insects challenged with these bacteria could result in the demonstrated changes in fat body lysozyme content and hemolymph antibacterial activity level depending on bacteria used for immune challenge.

It should be noted that pre-treatment of *E. coli*- but not *M. lysodeikticus*-challenged larvae with PKA inhibitor caused changes similar, to some extent, to those detected in LPS-injected larvae pre-treated with PKA inhibitors (Cytryńska *et al.,* 2006). The observed similarities could be a result of the LPS presence in *E. coli* cell walls and the differences probably reflect a more complex reaction of *G. mellonella* larvae to whole bacterial cells.

The presented results suggest the involvement of PKA in the humoral immune response of *G. mellonella* larvae to Gram-positive as well as Gram-negative bacteria. We demonstrated that injection of bacteria caused an increase in PKA activity in the fat body of *G. mellonella* larvae. The inhibition of the enzyme activity was correlated with changes in the fat body lysozyme content and antibacterial activity level in the hemolymph of bacteria-challenged insects. The observed changes were dependent on the bacteria used for immune challenge. This might suggest the participation of different pathways responding to both classes of bacteria in *G. mellonella* larvae. Whether it results in the appearance of a different set of antimicrobial peptides remains to be elucidated.

Acknowledgements

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