Signal Transduction for Cecropin B Gene Expression in Adherent Hemocytes of the Silkworm, *Bombyx mori* (Lepidoptera: Bombycidae)

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Expression of a cecropin gene of *Bombyx mori* (*B. mori*) in isolated hemocytes was triggered *in vitro* by lipopolysaccharide (LPS), dibutyrylcyclic AMP (dcAMP), *Vibrio cholerae* (V.c.) toxin, ionomycin and 4 β -phorbol 12-myristate 13-acetate (PMA), suggesting that cyclic AMP (cAMP), G protein, Ca²⁺ and protein kinase C (PKC) might be involved in expression of the gene. Inhibitors such as H-7 and H-89, but not genistein, greatly suppressed cecropin gene activation by LPS, strongly suggesting that PKC and protein kinase A (PKA), but not tyrosine kinases, may participate in the signal transduction. Conversely, other serine/threonine kinase inhibitors such as W-7 and KN-62 showed only weak suppression. This result suggested that myosin light chain kinase and Ca²⁺/calmodulin dependent protein kinase did not play a significant role in cecropin B gene activation. The inhibition of the induction by cycloheximide indicated that a certain protein factor with a high turnover rate may be indispensable for cecropin B gene expression triggered by LPS.

Key words: cecropin, gene expression, lipopolysaccharide, signal transduction, protein kinases

INTRODUCTION

Lipopolysaccharide (LPS) is a cell wall component of Gram-negative bacteria and has been known as a bacterial endotoxin, an exogenous pyrogenic substance in mammals. LPS has been demonstrated to modulate a spectrum of immunologically relevant responses. For example, in the mammalian immune system, LPS can affect T-cell responses, initiate B cell proliferation and antibody production, and activate macrophages to become tumoricidal and to release the colony stimulating factors, interleukin 1, interferons and tumor necrosis factor (MORRISON and RYAN, 1979). In the case of insect immunity, LPS has been proved to have multiple physiological effects such as the enhancement of phagocytosis (RATCLIFFE et al., 1984), activation of the prophenoloxidase cascade (SAUL and SUGUMARAN, 1987), induction of antibacterial proteins (ANDERSON and COOK, 1979), induction of nodule formation (GUNNARSSON, 1985) and change of hemocyte number (DUNPHY and WEBSTER, 1988).

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Concerning the induction of insect self-defense proteins by the effector molecules, the following results have been reported: 1) Gene expression of acidic and basic attacins of the giant silkworm, *Hyalophora cecropia* (Lepidoptera) is strongly induced by LPS, 4 β -phorbol 12-myristate 13-acetate (PMA) and bacteria (SUN et al., 1991 a). 2) LPS and β -1,3-glucan, but not PMA or tumor necrosis factor α , can activate both sarcotoxin II gene and lectin gene of the flesh fly, *Sarcophaga peregrina* (Diptera) (KOBAYASHI et al., 1993). 3) The cecropin gene expression of *Drosophila melanogaster* (Diptera) is induced by LPS, β -1,3-glucan and bacterial flagellin (SAMAKOVLIS et al., 1993). In the case of *D. melanogaster*, results of experiments with different drugs suggest that G protein, but not protein kinase C (PKC) or eicosanoids, is involved in cecropin gene expression, and that a protein factor(s) with a high turnover rate is required for the gene expression (SAMAKOVLIS et al., 1993).

In order to better understand the self-defense mechanism in insects, we attempted to characterize the mechanism of signal transduction in activation of the cecropin B gene using another lepidopteran insect, the silkworm, *Bombyx mori* (*B. mori*). In the present study, we focussed on the possible components such as protein kinases and other factors involved in cecropin gene expression. The findings suggest that PKC and protein kinase A (PKA) may play important roles in cecropin B gene activation triggered by LPS, and that a G protein, Ca²⁺ and cyclic AMP (cAMP) may be involved in induction of the gene expression.

MATERIALS AND METHODS

Materials. LPS (*Escherichia coli* serotype 0111:B4) was obtained from List Biological Laboratories. Serine/threonine kinase inhibitors (H-7, H-89, W-7 and KN-62) and a tyrosine kinase inhibitor (genistein) were purchased from Seikagaku Corporation and Wako Pure Chemical Co., respectively. Cycloheximide, PMA and dibutyryl cyclic AMP (dcAMP) were obtained from Sigma Chemical Co. *Vibrio cholerae* (V.c.) toxin and ionomycin were purchased from Calbiochem and Novabiochem Corporation. Insect cell culture medium powder, TC-100, was the product of GIBCO Laboratories. [α -³²P] dCTP was obtained from ICN Biochemicals, Inc. LPS was freshly prepared in LPS-free saline (Otsuka Pharmaceutical Co.) just prior to use. For stock solutions, H-7, genistein, V.c. toxin and dcAMP were dissolved in distilled water; PMA, ionomycin, H-89, W-7 and KN-62 were dissolved in DMSO and cycloheximide in ethanol. Working solutions were made in TC-100 media just prior to use. For a TC-100 stock solution, TC-100 powder supplemented with NaHCO₃ (0.35 g/l) was dissolved in distilled water and the pH was adjusted to 6.2. This solution was filtrated for sterilization with a 0.22 μ m Millipore filter. Phenylthiourea (0.1%, w/v) was added to the media to suppress activation of the prophenoloxidase cascade.

Insect and primary cell culture. Silkworms, *B. mori* (Tokai $\times$ Asahi), were reared on an artificial diet (Nihonnosanko, Co.) at 25°C. Fifth instar larvae (4 days) of *B. mori* were disinfected by immersion in 70% ethanol for 10 min. Hemolymph was then collected by cutting the abdominal legs into a plastic culture plate (diameter 60 mm, Becton Dickinson and Company) containing TC-100 medium with 0.1% phenylthiourea. The plates were kept for 30 min at room temperature, the supernatant was discarded and the adherent cells, comprised of granulocytes and plasmatocytes (WAGO, 1980), were washed twice with TC-100 media. Hemocytes were cultured in an incubator at 26°C until used.

RNA extraction and Northern blotting. Hemocyte primary culture cells (10⁵⁻⁶ cells/plate) were incubated with or without various stimuli in an incubator at 26°C and total RNA of adherent hemocytes was then extracted by the phenol-chloroform method (SAMBROOK et

al., 1989) using an Isogen kit (Nippon Gene Co.). Briefly, adherent cells were dissolved in 0.5 ml Isogen solution containing phenol and guanidine thiocyanate and collected by a cell scraper into a polypropylene test tube. After vigorous homogenization for 30 s with Physcotron (Nichionirikaki Co.), total RNA was extracted in a stepwise manner with chloroform, isopropanol and ethanol. Twenty to 30 μ g of total RNA was obtained from the hemocytes of 8 larvae. The RNA sample (10 μ g/lane) was electrophoresed on a 1.2% agarose gel containing 6.6% formaldehyde and transferred onto a Gene Screen plus membrane (Dupont-New England Nuclear). 32 P-labeled cDNA of cecropin B (BmcecB 19, KATO et al., 1993) was used as a probe for the analysis of cecropin B gene expression, and 32 P-labeled *B. mori* α -cytoplasmic actin DNA (MOUNIER and PRUDHOMME, 1991) was employed as a probe for an internal marker. Hybridization was carried out under highly stringent conditions, as previously described (KATO et al., 1993). The membrane was washed twice at room temperature with $2 \times$ SSC and once at 60°C with $0.2 \times$ SSC containing 0.5% SDS and autoradiographed on an X-ray film at -80°C overnight. In some cases, the membrane was rehybridized with a 32 P-labeled α -cytoplasmic actin probe after stripping off the cecropin probe in the solution containing 5 mM Tris-HCl buffer (pH 8.0), 0.2 mM EDTA, 0.05% sodium pyrophosphate, 0.002% polyvinylpyrrolidone, 0.002% bovine serum albumin and 0.002% ficol with constant agitation at 70°C for 16–20 h.

RESULTS

Induction kinetics of cecropin B gene expression in isolated B. mori hemocytes

In *B. mori*, cecropin B gene is expressed tissue-specifically in fat bodies and hemocytes (KATO et al., 1993). We first examined, by Northern blot analysis, whether LPS could induce cecropin gene expression in isolated hemocytes. Figure 1 shows the effects of induction time (Panel A) and LPS concentration (Panel B) on the expression of cecropin B gene. Cecropin gene expression was clearly seen 1 h post incubation, continued until 6 h post incubation and diminished to the background level by 12 h under experimental conditions employed here. The initiation of cecropin gene expression was observed with 0.01 μ g/ml LPS and the expression reached a maximum level at 1 μ g/ml.

Effects of various drugs on cecropin B gene expression

Various drugs such as cycloheximide, PMA, dcAMP, V.c. toxin and ionomycin were used to analyze the induction mechanism of cecropin B gene expression (Fig. 2). Pre-incubation of isolated hemocytes for 2 h with cycloheximide showed a significant inhibition of the induction by LPS, suggesting that *de novo* protein synthesis is required for the induction (Fig. 2, Panel A). Although ethanol (used as a vehicle of cycloheximide) was added to the hemocyte culture as a control, no cecropin B gene expression was observed (Fig. 2, Panel A). Drugs such as PMA, dcAMP, and ionomycin and V.c. toxin could efficiently induce cecropin B gene expression (Fig. 2, Panel B). On the contrary, in control experiments where DMSO (used as a vehicle of PMA and ionomycin) or H₂O (used as a vehicle of V.c. toxin and dcAMP) was added to the culture, cecropin B gene expression was not observed at all (Fig. 2, Panel B). These results suggested that PKC, cAMP, a G protein and Ca²⁺ might be involved in the gene expression.

Effects of PKC depletion, H-7, H-89 and genistein on cecropin B gene expression

Using the *in vitro* system, we tested whether protein kinases other than PKC partici-

Fig. 1. Induction kinetics of cecropin B gene expression in *B. mori* adherent hemocytes *in vitro*. Cecropin B gene expression was assayed by Northern blot analysis. *B. mori* hemocytes (10^6 cells/plate) were incubated with LPS ($1 \mu\text{g/ml}$) for the indicated time periods (A) or the cells were treated for 1 h with LPS at the indicated concentrations (B). After extraction from hemocytes using an "Isogen" kit, total RNA ($10 \mu\text{g/lane}$) was electrophoresed, blotted onto a "Gene Screen" membrane and hybridized with ^{32}P -labeled cecBm19 cDNA.

pated in the signal transduction pathway of cecropin B gene activation. To deplete endogenous PKC, isolated hemocytes were exposed to 100 nM PMA for 20 h and then to DMSO for 2 h. Northern blot analysis showed a weak but clear signal (Fig. 3, lane 1), suggesting that under such conditions, PKC was not completely depleted or other enzymes were involved in the cecropin gene activation. We incubated the hemocytes with PMA for 20 h and then treated the cells continuously with freshly added PMA for an additional 2 h. Northern blot analysis revealed that cecropin gene expression was very weak (Fig. 3, lane 3), suggesting that once hemocytes were treated with PMA for a prolonged period, re-addition of the same drug failed to activate the cecropin gene expression further. However, when we incubated the cells with DMSO for 20 h and then with PMA for 2 h, the cecropin gene was fairly activated and a much stronger signal was detected (Fig. 3, lane 2). In this case, DMSO did not appear to contribute to the accumulation of cecropin gene transcripts, since a control experiment, where hemocytes were incubated with DMSO alone, did not induce cecropin B gene expression (Fig. 2). These results suggested that prolonged treatment of the cells with PMA dramatically reduced endogenous PKC in the hemocytes. In the next step, hemocytes were incubated successively with 100 nM PMA for 20 h, with 0.01% DMSO for 2 h and finally with LPS ($1 \mu\text{g/ml}$) for 1 h. Northern blot analysis showed that the intensity of the signal (Fig. 3, lane 5) was stronger than that of the no LPS-treated control sample (Fig. 3, lane 1), but weaker than that of the hemocyte sample treated sequentially with DMSO and LPS (Fig. 3, lane 4). The results again suggested that either PKC was not completely depleted under the present experimental conditions, or a factor(s) other than PKC is involved in the signal transduction pathway. To test the latter possibility, the effects

Fig. 2. Effects of signaling modulators on cecropin B gene expression. *B. mori* adherent hemocytes (10^5 cells/plate) were first preincubated with $118 \mu\text{M}$ cycloheximide for the indicated times and then further incubated with LPS ($1 \mu\text{g/ml}$) (Panel A). As a control, ethanol was added to the hemocyte culture instead of cycloheximide (Panel A). Similarly, the hemocytes were incubated for 1 h with PMA (1 nM), dcAMP (1 nM), V.c. toxin ($0.5 \mu\text{g/ml}$) and ionomycin (10 nM) (Panel B). As controls, DMSO or H_2O was added to the hemocyte culture and cecropin B gene expression was examined under the same conditions described in Fig. 1 (Panel B). As an internal marker, actin gene transcripts were analyzed with *B. mori* α -cytoplasmic actin probe (lower panel).

of protein kinase inhibitors such as H-7, H-89 and genistein on cecropin gene activation by LPS were observed under the same conditions. H-7 and H-89 strongly inhibited gene activation by LPS, but genistein did not (Fig. 3, lanes 6–8). These results implied that LPS might activate cecropin B gene through pathways involving PKC and PKA but not tyrosine kinases.

Effects of KN-62 and W-7 on cecropin B gene expression

We examined the possibility of the involvement of other protein kinases in the signal

Fig. 3. Effects of PKC depletion, H-7, H-89 and genistein on cecropin B gene expression. *B. mori* adherent hemocytes (10^5 cells/plate) were first preincubated for 20 h with 0.01% DMSO (–, lanes 2 and 4) or with 100 nM PMA (+, lanes 1, 3, and 5–8) and the cells were then treated for 2 h with 0.01% DMSO (lanes 4 and 5), 100 nM PMA (lanes 2 and 3), 25 μ M H-7 (lane 6), 25 μ M H-89 (lane 7) or 25 μ M genistein (lane 8). After these treatments, the hemocytes were further stimulated for 1 h with 1 μ g/ml LPS (lanes 4–8). Northern blot analysis was performed with 32 P-labeled cecBm 19 cDNA (upper panel) and 32 P-labeled α -cytoplasmic actin probe (lower panel), as described in Fig. 1.

transduction using KN-62 and W-7, which are specific inhibitors for Ca^{2+} /calmodulin dependent kinase and myosin light chain kinase, respectively. KN-62 and W-7 slightly suppressed cecropin gene expression (Fig. 4, lanes 2 and 3). The inhibitors were confirmed to strongly suppress the activity of the relevant kinases (data not shown). Thus, the results suggested that possibility neither Ca^{2+} /calmodulin-dependent kinase nor myosin light chain

Fig. 4. Effects of KN-62 and W-7 on cecropin B gene expression. *B. mori* hemocytes (10^5 cells/plate) were preincubated for 2 h with 0.01% DMSO (lane 1), 25 μ M KN-62 (lane 2) or 25 μ M W-7 (lane 3) and subsequently stimulated for 1 h with 1 μ g/ml LPS (lanes 1–3). Northern blot analysis was carried out as described in Fig. 1.

kinase plays a significant role in the signal transduction for the LPS-triggered activation of cecropin B gene.

DISCUSSION

In this report, we demonstrated that cecropin B gene expression occurred in isolated adherent hemocytes, containing mainly granulocytes and plasmatocytes. Results of the present study using the *in vitro* system suggest the possible involvement of PKC and PKA rather than tyrosine kinases, myosin light chain kinase and Ca^{2+} /calmodulin dependent protein kinase in the activation of cecropin B gene expression triggered by LPS. The results also suggest that G protein, cAMP and Ca^{2+} might be involved in the signaling pathways of the gene expression. As for the results of experiments using dcAMP and ionomycin, however, the relationship between LPS and the increase of the endogenous level of cAMP and Ca^{2+} still remains unclear.

Concerning the induction of insect self-defense protein gene expression including cecropin genes by an effective stimulus such as LPS, at least two different possible pathways for intracellular signaling were suggested here. One is a PKC-mediated pathway in a lepidopteran insect, *H. cecropia* (SUN et al., 1991 a), and the other is a PKC-noninvolved pathway in two dipteran insects, *D. melanogaster* (SAMAKOVLIS et al., 1992) and *S. peregrina* (KOBAYASHI et al., 1993). Our results suggest that the signal transduction for *B. mori* cecropin gene activation by LPS belongs to the former pathway. There is probably a common pathway in lepidopteran insects which differs from that in dipteran insects. However, in the case of *D. melanogaster*, G protein has been demonstrated to participate in the cecropin gene induction (SAMAKOVLIS et al., 1992), implying that there should also be a common signaling

factor in both dipteran and lepidopteran insects. In addition, NF- κ B (LENARDO and BALTIMORE, 1989) binding site-related motifs have been demonstrated in the gene analysis of insect self-defense proteins such as *B. mori* cecropin (TANIAI et al., 1995), lysozyme (SUN et al., 1991 b) and attacins (SUN et al., 1991 a) of *H. cecropia*, cecropin (KYLSTEN et al., 1990) and dipterin (MEISTER et al., 1994) of *D. melanogaster* and Sarcophaga lectin and Sarcotoxin I and II of *S. peregrina* (KOBAYASHI et al., 1993). The motifs were shown to be important for the activation of self-defense protein genes by LPS (ENGSTRÖM et al., 1993; MEISTER et al., 1994) and the binding proteins to the motifs have been isolated (SUN and FAYE, 1992 a, b; KOBAYASHI et al., 1993). These results strongly suggest that insect self-defense protein genes contain a common regulatory element(s). Thus, we speculate that, although lepidopteran and dipteran insects may share at least a part of the factors or gene regulatory elements which are involved in the activation of their self-defense protein genes, some aspects of the signal transduction may be different, reflecting evolutionary divergence in insect immunological processes.

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