PUBLICATIONS
Identification and Characterization of a Novel Polypeptide in the CNS of *Bombyx mori* During Pupal - Adult Transformation

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Abstract: Detailed one and two dimensional electrophoresis (PAGE) studies carried out on the CNS proteins of *Bombyx* revealed the appearance of a new nervous system specific acidic polypeptide with a molecular mass of 235 kDa in the pharate adults. This polypeptide is totally absent in the late larval, early pupal and mid pupal stages. Synthesis of this polypeptide begins in pharate adults and its concentration increases gradually during the adult development and it reaches a very high concentration in 48 h old moths.

Keywords: Metamorphosis; cell death, nervous system protein; *Bombyx mori*

INTRODUCTION

Naturally occurring cell death or programmed cell death is an important feature of developing and metamorphosing nervous system of insects which regulates the size of neuronal population (Truman, 1984; Booker and Truman, 1987; Fahrbach and Truman, 1987). Cell death is required to eliminate the cells which are no longer required as well as for the generation of new cells having specific characteristics. The steroid hormone 20-hydroxyecdysone (20-HE) plays an important role in controlling post-metamorphic neuronal death in *Manduca sexta* (Truman and Schwartz, 1984). Furthermore, *in vivo* and *in vitro* studies using RNA and protein synthesis inhibitors reveal that *de novo* synthesis is required for the post-embryonic neuronal death (Fahrbach and Truman, 1987). The same has been found to be true for the programmed cell death in the intersegmental muscles in *Manduca* (Schwartz et al., 1990).

There is little doubt that much progress has been made towards understanding the process of programmed cell death in insects and significantly towards the actual role of metamorphic hormone (20-HE) in this process. Several approaches are currently being pursued. These include attempts to find mRNAs and/or proteins whose increased expression/synthesis is associated with cell death. Recently, a 40 kDa protein has been identified and implicated with neuronal death in *Manduca* (Montemayor et al.).

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In the present study an attempt has been made to analyse the pattern of CNS proteins in Bombbyx mori using one and two dimensional polyacrylamide gel electrophoresis (PAGE) and silver staining during pupal-adult transformation. At this time, the CNS contains a mixture of neurons that will survive during adult life, neurons that are already degenerating, and neurons that are committed to die but have not yet begun the process of degeneration (Truman, 1983; Truman and Schwartz, 1984). Thus, any changes in protein pattern detected at this time could reflect changes in protein expression in any of these three cell populations.

MATERIALS AND METHODS

Insects:

Third instar larvae of Bombbyx mori (pure Mysore strain) were obtained from local breeding centre and reared in an insect culture room at 26 ± 1°C temperature, 70 ± 5% relative humidity and 14 h: 10 h light-dark period on fresh mulberry leaves. For the present study, late-last instar larva, pre-pupa, mid-pupa, pharate adult (late-pupa) and adult stages were used.

Preparation of tissue sample:

The intact nervous system and other tissues - muscle, alimentary canal and salivary glands were rapidly dissected out and homogenized in chilled homogenization buffer (10 mM Tris; 0.1% Triton X-100; pH 7.1) using a glass microhomogenizer (Kontes). The homogenate was centrifuged at 1000 x g for 2-3 min in a microfuge at 4°C. The supernatant was collected and used for protein estimation and one dimensional electrophoresis. Before electrophoresis, sample was mixed with an equal amount of 2x sample buffer (containing 0.125 M Tris-HCl, pH 6.8; 4% SDS; 20% glycerol; 10% 2-mercaptoethanol and 0.002% bromophenol blue) and incubated at 100°C for 1 min. For two-dimensional electrophoresis, the tissue was homogenized in IEF sample buffer containing 9.5 M urea, 2% LKB carrier ampholytes (comprised of 1.6% 5/7 pH and 0.4% 3.5/10 pH), 2% NP-40 and 5% 2-mercaptoethanol.

Electrophoresis:

One dimensional SDS-PAGE was carried out according to the procedure of Laemmli (1970). A 1 cm 3.3% stacking gel (pH 6.8) was followed by a 15 cm separating gel (pH 8.8). Tris-glycine buffer (0.025 M) with 0.1% SDS (pH 8.3) was used as the first electrode buffer. Proteins were visualized by silver staining (Blum et al., 1987). Two dimensional gel electrophoresis was performed as described by O’Farrell (1975). Ampholyte polyacrylamide tubes were prefocused for 1 h at 200 V to set-up the pH gradient. The gels were run for a total of 10,000 V h. The anolyte used was 0.01 M H₃PO₄, the catholyte used was 0.02 M NaOH. The gels were subsequently transferred to 5 ml equilibration buffer containing 10% glycerol, 5% 2-mercaptoethanol, 2.3% SDS and 0.0625 M Tris-HCl (pH 6.8) and stored at -20°C. The second dimension separation was carried out using 3.3% stacking gel and 10% separating gel. High molecular weight (Sigma) standards were run at the acidic end of several gels. The gels were run at 25mA/gel until the bromophenol blue dye reach the bottom of the gel. The gels were fixed and silver stained. To determine the pH gradient of IEF gels, parallel gels were cut into pieces of 0.5 cm length and incubated for 2 h in 0.5 ml degassed
Fig 1 shows the polypeptide pattern from the CNS of late last instar larval (lane 1), mid-pupa (lane 2) and adult (lane 3). The protein was separated on a 10% SDS-PAGE, and in each lane 10 μg protein was loaded. Note the presence of a 235 kDa polypeptide in the CNS of adult moths. 

Fig 2 shows the polypeptide pattern in the CNS during various stages of development. 10 μg protein sample from each stage was separated on a 5% SDS-PAGE. The sample loaded in lane 1 is from pre pupa, lane 2 from early pupa, lane 3 from pharate adult (late-pupa), lane 4 from 12 h old adult, lane 5 from 24 h old adult, and lane 6 from 48 h old adult. Note that the concentration of the 235 kDa polypeptide increases in the CNS during the pupal and adult development. Lane 7 shows high molecular weight markers (Sigma). 

Distilled water. The pH was measured electrometrically. Densitometric scanning of one dimension gels were done with a Bio-Med soft laser scanning densitometer. For all studies equal quantity of protein samples were loaded and comparisons were made. Protein was estimated according to the method of Bradford (1976) in the case of samples for one dimensional electrophoresis and according to the method of Ramagli and Rodriguez (1985) for two dimensional electrophoresis, which allows the quantitation of the proteins in the presence of urea and carrier ampholytes.

RESULTS

Detailed electrophoretic (SDS-PAGE) studies carried out on the CNS proteins of *Bombyx* during pupal-adult transformation revealed the appearance of a new nervous system specific polypeptide with an apparent molecular weight of 235 kDa in pharate
A novel polypeptide in the CNS of Bombyx mori

Figs 3, 4 and 5 - Photographs showing the fractionation of CNS proteins from late-last instar larva (Fig. 3), early-pupa (Fig. 4) and adult (Fig. 5) on two dimensional polyacrylamide gel. The proteins were separated between 20 to 260 kDa on vertical axis and 4.7 to 7.2 p/ on horizontal axis. Note the presence of a new protein (>) which appears in the CNS of adult insect.

Figs 3, 4 and 5 - Photographs showing the fractionation of CNS proteins from late-last instar larva (Fig. 3), early-pupa (Fig. 4) and adult (Fig. 5) on two dimensional polyacrylamide gel. The proteins were separated between 20 to 260 kDa on vertical axis and 4.7 to 7.2 p/ on horizontal axis. Note the presence of a new protein (>) which appears in the CNS of adult insect.

adults. This polypeptide was totally absent in the late-last larval, prepupal and early and mid-pupal stages (Fig. 1, lanes 1 & 2; Fig. 2, lanes 1 & 2). Laser scanning densitometry of dried gels indicated that this polypeptide was present in low concentration in pharate adult (Fig. 2, lane 3) and its content gradually increases during adult development and reaches highest in 48 h old moths. The concentration of this protein remains more or less the same up to 4-5 days (which is the total life span of the adult moth). All the experiments were repeated thrice with tissue samples from three different batches. Subsequent analysis of CNS proteins by two dimensional electrophoresis clearly showed that this 235 kDa polypeptide was expressed only during the pharate
adult and adult development (Fig. 5) and it is totally absent during the larval (Fig. 3) and early pupal (Fig 4) development. The p/ value of the polypeptide ranges between 6-6.2. Furthermore, this polypeptide is found to be absent in other tissues like muscles, salivary glands and alimentary canal (data not presented) of larval, pupal and pharate adult stages.

**DISCUSSION**

Programmed cell death has been observed during the development of virtually all metazoan organisms. Extensive studies in *C. elegans* suggest that highly regulated genetic programme is responsible for programmed cell death and the proteins encoded by ced-3 and ced-4 genes act within the dying cells themselves and/or interact with other intracellular molecules to produce cell death (Ellis et al., 1991). While another gene ced-9 product, acts antagonistically to ced-3 and ced-4 products to suppress the cell death (Fanidi and Evan, 1994). DNA fragmentation has been demonstrated in some models of excitotoxin and neurotoxin induced cell death (Dispaquale et al., 1991; Kure et al., 1991) as well as programmed neuronal death (Clarke and Hornung, 1989; Johnson et al., 1989). Furthermore there increasing evidence for the possible role of nucleases in DNA fragmentation during neuronal death (Ashwell et al., 1994).

In this study we have identified a novel 235 kDa acidic polypeptide in the CNS of *Bombyx mori*, which shows a precise developmental regulation. The synthesis of the polypeptide begins at pharate adult stage and it may play a role in programmed cell death. Montemayor et al., (1990) have reported the appearance of a 40 kDa acidic protein in *Manduca* during neuronal death. However, the apparent molecular weight of the newly synthesized polypeptide in *Bombyx* seems to be nearly six times greater than that reported for *Manduca*. Programmed cell death in the intersegmental muscles of *Manduca* is shown to be suppressed by inhibitors of macromolecular synthesis indicating that it requires new RNA and protein synthesis (Lockshin, 1969; Schwartz et al., 1990).

In conclusion, in the present study we have demonstrated the expression of a developmentally regulated new tissue specific polypeptide which may play a role in CNS remodelling during metamorphosis of *Bombyx mori*. Further studies on identification of protein and its cellular distribution should provide an insight into whether its expression is actually related to the commencement of neuronal death.

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Dear Dr. Murthy:

I am pleased to inform you that your above manuscript is accepted for publication in J. Neurochem. I will send the revised manuscript to Chief Editor, which will be forwarded to Raven Press.

Yours sincerely,

Eishichi Miyamoto, M.D.

EM:ro
Identification, Characterization, Immunocytochemical Localization and Developmental Changes in the Activity of Calcium/calmodulin-Dependent Protein Kinase II in the Central Nervous System of *Bombyx mori* during Postembryonic development

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**Abstract:** In the present investigation *in vitro* phosphorylation of CNS proteins of the silkworm, *Bombyx mori* during the postembryonic development have been studied. SDS-PAGE and autoradiography of phosphorylated proteins, revealed the presence of major phosphoproteins of 59/60 kDa. Based on molecular mass, calcium/calmodulin-dependent autophosphorylation, substrate specificity, KN-62 inhibition, apparent Km for ATP and syntide-2, these proteins were identified as calcium/calmodulin-dependent protein kinase II (CaM kinase II). Anti-rat CaM kinase II monoclonal antibody showed immunoreactivity with *Bombyx* CaM kinase II isoforms. This kinase showed a high degree of autophosphorylation in neural tissue. During postembryonic development of *Bombyx mori*, two distinct peaks of enzyme activity could be noticed - one at the late larval and another at late pupal stage, which were associated with an increase in amount of the enzyme. These results suggested that the expression of CaM kinase II in the CNS of *Bombyx mori* was developmentally regulated.

**Running title:** *Bombyx* CaM kinase II

**INTRODUCTION**

Protein phosphorylation is now recognized to be a major regulatory mechanism by which neural activities are controlled by external physiological stimuli (Cohen, 1982; Browning et al., 1985). Considerable evidence now indicates that most of the effects of cAMP, cGMP as well as many of the effects of calcium involve activation of specific protein kinases. There are two types of cAMP-dependent protein kinase and cGMP-dependent kinase in brain, while there are a number of different calcium-dependent protein kinases. Multiple Ca\(^{2+}\)/calmodulin-dependent protein kinases and Ca\(^{2+}\)/phospholipid-dependent protein kinases (PKCs) have been identified in brain (Rosen and Krebs, 1981; Nestler and Greengard, 1984; Nairn et al., 1985; Hanson and Schulman, 1992; Hidaka and Ishikawa, 1992).

**Abbreviations used:** CaM kinase, calcium/calmodulin-dependent protein kinase II; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; DTT, Dithiothreitol; NBT, nitroblue tetrazolium; BCIP, 5-bromo-4-chloro-3-indolyl phosphate; BSA, bovine serum albumin; TBS, tris-buffered saline.
Analysis of the physiological functions of CaM kinase II has drawn considerable attention over the last few years. This kinase is a multifunctional mediator of the activity dependent calcium release in excitable cells, which has been associated with physiological and behavioural plasticity in both vertebrates (Malenka et al., 1989; Malinow et al., 1989; Silva et al., 1992a, b; Rotenberg et al., 1996; Strack et al., 1997a) and invertebrates (Wilmund et al., 1986; Griffith et al., 1993; Wang et al., 1994).

Rat brain CaM kinase II comprises of several related isozymes (for review see Hanson and Schulman, 1992). The rat isozymes consists of a catalytic domain, an autoregulatory domain containing a calmodulin-binding site and C-terminal "association domain" that mediates holoenzyme formation (Bennet and Kennedy, 1987; Tobimatsu and Fujisawa, 1989). They are encoded by separate genes and differ mainly in a region between the regulatory and association domains (Hanson and Schulman, 1992). A distinct property of CaM kinase II is that autophosphorylation of its threonine residue near calmodulin binding domain converts it to Ca\(^{2+}\) independent state (Miller and Kennedy, 1986) Further, it was shown that calmodulin-dependent autophosphorylation of CaM kinase II induces a conformational change in the region of the calmodulin binding domain which allows additional stabilizing interactions with calmodulin (Putkey and Waxham, 1996). It has been postulated that this autoregulation may be involved in prolonging the effects triggered by transient calcium signal (Miller and Kennedy, 1986) and also may induce its translocation in Aplysia and Drosophila cells (Saitoh and Schwartz, 1985; Wilmund et al., 1986).

The adult Drosophila head contains three isoforms of CaM kinase II with molecular mass of 54, 58 and 60 kDa (Cho et al., 1991; Ohsako et al., 1993). These cross-react with anti-rat CaM kinase antibody. These isoforms are generated from a single gene by alternate splicing (Ohsako et al., 1993; Griffith and Greenspan, 1993). Both amino acid sequence and tissue specificity of the rat kinase are highly conserved in Drosophila (Cho et al., 1991).

With the exception of Drosophila, there are no reports on the characterization of CaM kinase II of other insects. Here we report our studies on the CaM kinase II of Bombyx mori, that has many of the characteristics of the rat and Drosophila CaM kinase II including high level of activity in the neural tissue and regulation by autophosphorylation. In addition we also demonstrate changes in the activity and content of this enzyme in the CNS during larval-pupal-adult transformation.

**MATERIALS AND METHODS**

**Animals:** Third instar larvae of Bombyx mori (pure Mysore strain) were obtained from local breeding centre and reared in an insect culture room at 26 ± 1 °C, 70 ± 5% relative humidity and 14 h : 10 h light-dark period on fresh mulberry leaves. Staging of insects was done based on their age after the 4th ecdysis. One to 2 days old last instar larvae were designated as early-last instar (ELI), 5 to 6 days old as mid-last instar (MLI) and 9-10 days old as late-last instar (LLI). Larvae collected after spinning were designated as prepupa (PP). One to 2 days old pupa as early-pupa (EP), 4-5 days old as mid-pupa (MP) and 9-10 days old as late-pupa (LP). Freshly emerged moths (<12 h old) were used as adult (A).

**Materials:** [\(\text{P}^{32}\text{P}\)]ATP was obtained from BARC, Trombay, India. Syntide-2 (CaM kinase II substrate peptide), calmodulin, EGTA, DTT, diacylglycerol, phosphatydylserine and high molecular weight protein markers were obtained from Sigma Chem. Co. (St. Louis, MO).
Monoclonal rat anti-CaM kinase II a antibody was purchased from Boehringer Mannheim (Darmstadt, Germany). Okadaic acid was obtained from Gibco-BRL (USA). Alkaline phosphatase-conjugated secondary antibody, nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) were from Pierce (Rockford, IL). KN-62 was a gift from Dr. H. Hidaka, Nagoya University School of Medicine, Japan. Nitrocellulose sheets were from Millipore, USA. P81-phosphocephalose papers were from Whatman (Maidstone, UK). All other reagents were of highest grade and were obtained from local sources.

Preparation of tissue sample: Intact CNS (brain + ventral nerve cord) was rapidly dissected from larvae of different developmental stages, frozen in liquid nitrogen and stored at -70°C. Frozen tissue was homogenized (4 CNS/50 ul) by hand in 50 mM HEPES buffer, pH 7.4, containing 1 mM EDTA and 1 mM DTT using an all glass microhomogenizer (Kontes). Homogenates were centrifuged at 1000 g for 5 min to remove debris. Freshly prepared homogenates were used for phosphorylation reaction. Protein content was estimated according to the method of Bradford (1976).

Endogenous phosphorylation in Bombyx tissue homogenates: Incubations were carried out in 1.5 ml Eppendorf tubes in a total volume of 40 ul, containing 20 μg of CNS homogenate protein. Incubation mixture consisted of 50 mM HEPES, pH 7.4, 10 mM MgCl2, 1 mM DTT, 2 μM calmodulin, 10 mM ATP, 1 mM EGTA or 0.1 mM EGTA and 1 mM CaCl2. This was preincubated for 5 min at 30°C and the reaction was initiated by the addition of 4 μCi of [γ-32P]ATP (~ 3000 Ci/mm). After 1 min, reaction was terminated by the addition of 20 ul of 3x sample buffer (0.188 M Tris-HCl, pH 6.8, 6% SDS, 30% glycerol, 15% 2-mercaptoethanol and 0.003% bromophenol blue) and immersion in boiling water for 2 min. Following centrifugation at 10,000 g for 3 min, 30 ul of the supernatant (10 μg protein) was subjected to SDS-PAGE.

In vitro phosphorylation reactions were also carried out under appropriate conditions to study the effect of phosphatydylserine + diacylglycerol, KN-62 and okadaic acid.

Assay of CaM kinase II: CaM Kinase II activity was assayed by the phosphorylation of syntide-2 according to the method of Fukunaga et al., (1989). Reaction mixture contained 50 mM HEPES, pH 7.5, 10 mM MgCl2, 0.1 mM [γ-32P]ATP (2000-4000 cpm/pmol), 30 μM syntide-2, 2 μM calmodulin, 1 mM CaCl2, 0.1 mM EGTA and suitable amounts of homogenate protein in a total volume of 50 ul. Control reaction was carried out in the presence of 1 mM EGTA. After incubation at 30°C for 1 min, the reaction was terminated by adding 10 ul of 0.4 M EDTA and a 50 ul aliquot was spotted on phosphocellulose paper squares. Radioactivity was determined as described by Roskosky (1983). Assay conditions were standardized using CNS homogenates from freshly emerged adults.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography: [32P] labeled phosphoproteins in CNS homogenates were separated on 1 mm slab gels of 10% acrylamide using the system of Laemmli (1970). Equal amount of protein was loaded in all the wells. Gels were stained by the silver method of Blum et al., (1987). The stained gels were photographed, dried under vacuum between cellophane sheets using a Hoeffer Gel drier and exposed for 1-3 days to Kodak X-Omat AR film at -70°C.

Western blotting and immunostaining: Proteins were electrophoresed by SDS-PAGE and were electroblotted at 70 V for 3 h on to a nitrocellophose membrane using Trans Blot apparatus (BioRad) according to the procedure of Towbin et al., (1979). After the transfer, membrane was air dried and was then incubated for 1 h at room temperature with 3% (w/v) bovine serum albumin (BSA) in TBS (10 mM Tris, 150 mM NaCl, pH 7.5) to block non-specific binding.
sites. The blot was incubated overnight with monoclonal CaM kinase II antibody (10 μg/ml) in TBS containing 3% BSA. This was followed by a thorough wash in TBS (5 min x 6 changes). Thereafter, the blot was incubated with the alkaline phosphatase-conjugated anti-mouse IgG (goat) for 1 h. The blot was once again washed in TBS (5 min x 5 changes) and stained in 10 ml ALP buffer (10 mM Tris, 5 mM MgCl$_2$ and 100 mM NaCl, pH 9.5) containing 0.033% NBT and 0.0165% BCIP.

Tissue preparation and immunocytochemical staining: The CNS of freshly emerged adults was dissected in ice-cold 0.1 M TBS and fixed in Bouin's fixative for 8 h. Tissue was washed in TBS, dehydrated in ethanol, cleared in xylene, and embedded in paraffin. Ten micron thick sections were cut, rehydrated and were processed for immunocytochemical staining.

The sections were first incubated with 1% non-immune goat serum to block nonspecific binding sites. Then they were sequentially incubated with monoclonal anti-CaM kinase II antibody (10 μg/ml) for 24 h at 4°C; alkaline phosphatase-conjugated goat anti-mouse IgG (1:100) for 1 h. All antibodies were diluted in 50 mM TBS (pH 7.5) containing 1% non-immune goat serum. Each incubation with antibody was followed by three 10 min washes in TBS. The sections were finally stained in NBT/BCIP as described by Meltzer et al., (1997). Staining specificity was assessed by replacing the primary antibody with the IgG fraction derived from non-immune mouse serum. Slides were visualized and photographed with a Nikon Labophot II microscope.

Molecular weight determination and densitometric scanning: Molecular weights of electrophoretically separated polypeptides were determined by co-electrophoresing high molecular weight marker proteins. They included myosin (205 kDa), β-galactosidase (116 kDa), phosphorylase B (97.4 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa) and α-lactalbumin (14 kDa). Quantitation of autoradiograms and immunoblots was done using a computing laser scanning densitometer (Molecular Devices).

Statistical analysis: Statistical analysis was performed by one way ANOVA with Sigma Stat software. The data are represented as means ± S.D. Values are considered statistically significant only when $p < 0.05$.

RESULTS

In vitro phosphorylation of endogenous proteins of Bombyx CNS

In vitro phosphorylation of CNS proteins in presence of EGTA revealed the phosphorylation of few proteins, of which a 48 kDa band was the major protein labeled with $[^{32}P]$ (Fig. 1b, lane 1). Inclusion of 1 mM CaCl$_2$ stimulated the phosphorylation of several proteins, particularly 59/60 kDa (Fig. 1b, lane 2) and this stimulation was further enhanced by the addition of 2 μM calmodulin (Fig 1b, lane 3). However, PKC activators, phosphatidyserine + diacylglycerol had no effect on the phosphorylation of this protein (data not presented). This increase in phosphorylation of 59/60 kDa protein was inhibited by 80% when the homogenate was incubated for 5 min with 10 μM KN-62, a specific inhibitor of CaM kinase II (Fig. 1c, lane 2).

Identification of 59 and 60 kDa proteins as isoforms of CaM kinase II

Rat CaM kinase II a antibody was used to identify the CaM kinase II in the CNS homogenates of Bombyx and the results are presented in figure 2. Rat brain homogenate was used as control. The 50 kDa a subunit of rat CaM kinase II showed strong crossreactivity to the antibody (lane 2). Two closely migrating proteins of molecular weights 59 and 60 kDa from Bombyx CNS
homogenate cross reacted with the antibody (lane 1). Autoradiography of the immunoblot revealed \(^{32}P\) incorporation into these two bands. These bands were excised from the blot separately and the \(^{32}P\) incorporation was measured by liquid scintillation spectrophotometry. This confirmed the autophosphorylation of CaM kinase II. Since these two bands migrate closely, the signal on the autoradiograph appears as a single band. No other protein in the Bombyx CNS homogenate cross reacted with the antibody.

**Dephosphorylation of autophosphorylated CaM kinase II and the effect of okadaic acid**

Time course studies on the phosphorylation of major phosphoproteins revealed that maximal \(^{32}P\) incorporation was achieved by 1 min at 30° C. Of interest was the observation of rapid dephosphorylation of the autophosphorylated CaM kinase II with increase in reaction time. With 5 min of incubation time the \(^{32}P\) incorporation decreased by 1/5th of that at the end of 1 min (Fig. 3), suggesting the involvement of a protein phosphatase in this process. Okadaic acid (1 nM) had no effect on the dephosphorylation of the kinase (Fig. 3, lane 6) while higher concentrations (10 and 500 nM) inhibited the activity of the phosphatase in a dose dependent manner (Fig. 3, lanes 7 and 8).

**Catalytic properties of Bombyx CaM kinase II**

The rate of phosphorylation of the peptide substrate syntide-2 by CaM kinase II showed normal Michaelis-Menten kinetics with respect to the concentration of ATP and syntide-2 (Figs. 4a and b). In the presence of saturating amount of syntide-2, the \(K_m\) value for ATP was 21 \(\mu\)M. The synthetic peptide proved to be a very effective substrate for Bombyx CaM kinase II with an apparent \(K_m\) of 12.5 \(\mu\)M and a \(V_{max}\) of 2.68 nmol/min/mg homogenate protein.

**Changes in the activity of CaM kinase II in the CNS of Bombyx during larval-pupal-adult transformation**

CaM kinase II was assayed in the CNS homogenates from different developmental stages and the results are presented in figure 5. A comparison of the CaM kinase II activity revealed a continuous rise in the activity from early-last instar larvae to adult stage. However, at two stages of development, late-last instar and late-pupa, there was a sudden spurt in the activity when compared to the immediate previous stage (i.e., 47% difference between late-last instar and mid-last instar; 29% difference between late-pupa and mid-pupa). Immediately after this surge, there was a decrease in the activity (-25% between late-last instar and prepupa; -14% between late-pupa and adult).

**Developmental changes in the levels of CaM kinase II**

Determination of CaM kinase II activity was followed by the immunoblotting of equal amount of CNS protein extracts of various developmental stages to find out the correlation, if any, between the activity and quantity of the enzyme (Figs. 6a and b). This study revealed that changes in the levels of CaM kinase II at various developmental stages may be due to changes in the enzyme content. Amount of the enzyme protein was found to be maximal in late-pupal and lowest in the early-last larval stage.

**Immunocytochemical localization of CaM kinase II in the CNS of Bombyx**

Light microscopic study demonstrated more or less evenly distributed immunoreactivity in coronal sections of the adult brain. Cell bodies in the pars- intercerebralis and tritocerebrum showed intense staining when compared with the neuropil (Fig. 7b). In the giant neurons of the metathoracic ganglion of the adult, the cross reactivity was strong in the cytoplasm and absent in the nucleus (Fig. 8b).
DISCUSSION

In the present study CaM kinase II has been identified and characterized from Bombyx CNS. The kinase consists of two species with molecular weights of 59 and 60 kDa and both exhibited Ca\(^{2+}\) and calmodulin-dependent autophosphorylation. Drosophila head contains three major species of CaM kinase II with molecular masses of 54/55, 58, and 60 kDa, which cross reacted with anti-rat CaM kinase II antibody (Cho et al., 1991; Ohsako et al., 1993). However, in Bombyx we observed only two species although possibility of other isoforms of same molecular mass, migrating together in SDS-PAGE cannot be ruled out.

KN-62, a potent inhibitor of CaM kinase II has been extensively used in investigating the physiological role of this enzyme (Tokumitsu et al., 1990). It has been shown to inhibit the autophosphorylation of the kinase in a dose dependent manner. In the present study we have observed about 80% inhibition in autophosphorylation of the kinase with 10 µM KN-62.

Several lines of evidence suggest that protein phosphatase 1 (PP1) is more likely to be involved in the dephosphorylation of CaM kinase II in mammals than protein phosphatase 2A (PP2A) (Shenolikar and Nairn, 1991; Strack et al., 1997b). Protein phosphatase 2C has also been reported to be involved in the dephosphorylation of autophosphorylated CaM kinase II in rat cerebellar granule cells (Fukanaga et al., 1993). There are no reports on the identification of phosphatase involved in the dephosphorylation of CaM kinase II in insects. However, PP1 deficient mutant of Drosophila show impaired associative learning and visual conditioning (Asztalos et al., 1993). Okadaic acid has been extremely useful in identifying protein phosphatases involved in dephosphorylation of proteins (Biolojan and Takai, 1988). The activity of PP2A has been shown to be completely inhibited by 1 nM okadaic acid; however, PP1 activity is inhibited only at 1 µM okadaic acid and is resistant to 1 nM okadaic acid (Cohen et al., 1990). Our studies using okadaic acid suggested PP1 as the phosphatase involved in the dephosphorylation of autophosphorylated CaM kinase II of Bombyx.

The high degree of autophosphorylation of CaM kinase II in the neural tissue observed in the present investigation corroborates with earlier reports on Drosophila demonstrating high expression of CaM kinase II transcripts and its protein product in the head than in the body (Cho et al., 1991; Ohsako et al., 1993). Several studies have identified potential substrate proteins of CaM kinase II in vertebrates (Hanson and Schulman, 1992). Among insects, Combest and Gilbert (1986) have reported the phosphorylation of 42 and 25 kDa protein in the brain of tobacco hornworm, Manduca sexta, which was stimulated by Ca\(^{2+}\) and calmodulin. Recently, the photoreceptor-specific protein, Phosrestin 1 has been identified as a substrate of this kinase in Drosophila (Kahn and Matsumoto, 1997).

Studies on the catalytic properties of Bombyx CaM kinase II revealed that \(K_m\) values for ATP and syntide-2 are close to the values reported for rat and Drosophila (Hashimoto and Soderling, 1987; Ohsako et al., 1993). Developmental changes in CaM kinase II is well documented in vertebrates with noted changes in enzyme activity, subunit ratios, mRNA levels and subcellular localization during neuronal development (Rostas, 1991; Sugiura and Yamauchi, 1994). Rat forebrain shows a 10 fold increase in CaM kinase II mRNA between day 1 and 21 and further 2 to 5 fold increase by day 90 (Hanley et al., 1987). The increase in a subunit levels peaks near the end of the most active period of synaptogenesis (Kelly et al., 1987) and may be coincident with synaptic maturation (Rostas, 1991). An interesting observation in the present study was the presence of two peaks in the activity of the enzyme - first one at the late-larval stage and the next peak at the late-pupal stage. It is interesting to note that parallel changes were reported in the ecdysteroid titre in the haemolymph of B. mori during postembryonic development (Calvez et al., 1976). Further studies are required to
understand the functional significance of these changes in the activity. Immunoblot analysis of the kinase from different developmental stages indicates that variations in the enzyme activity may be accounted to some extent by changes in the enzyme content. These results suggest a developmental regulation in the expression of CaM kinase II in the CNS of Bombyx during metamorphosis. Induction of cellular differentiation in neuroblastoma/glioma cell cultures leads to a significant increase in enzyme activity as well as enzyme content, suggests a clear role for CaM kinase II in the process of development and differentiation of nerve cells (Vallano and Hall, 1989).

Immunocytochemical localization of CaM kinase II in rat brain revealed differences in its regional distribution with the highest concentration in the hippocampus (Ouimet et al., 1984; Fukanaga et al., 1988). There are no reports on the distribution of CaM kinase II protein in insect brain and the results of present study revealed strong staining of cell bodies when compared to the neuropil. In the neurons of the metathoracic ganglion of the adult, immunoreactivity was very strong in the neuronal cytosol and absent from the nucleus.

At least four CaM kinase II genes have been identified in rat whereas only one gene has been identified in Drosophila (Cho et al., 1991; Ohsako et al., 1993) The CaM kinase II gene generates at least 8 transcripts in Drosophila (Griffith and Greenspan, 1993), suggesting that requirement for functional complexity in the gene products is compensated by alternate splicing. In general the properties observed for Bombyx CaM kinase II in this report appear to be similar to the rat and Drosophila CaM kinase II, supporting the concept that CaM kinase II has been highly conserved during evolution.

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Fig. 4a

\[ \frac{1}{[V]} \times 10^{-4} \text{ (pmol/min/mg protein)} \]

$V_{\text{max}} = 1.54 \text{ nmol/min/mg protein}$

$K_m = 21.3 \mu M$

Fig. 4b

\[ \frac{1}{[ATP]} \times 10^{-3} \text{ [\mu M]}^{-1} \]

$V_{\text{max}} = 2.68 \text{ nmol/min/mg protein}$

$K_m = 12.5 \mu M$