PART - II

STUDIES ON PROTEIN PHOSPHORYLATION SYSTEMS IN THE CENTRAL NERVOUS SYSTEM OF *BOMBYX MORI* DURING POSTEMBRYONIC DEVELOPMENT
The presence of phosphorus in proteins has been known for almost 100 years, but the importance has only been realized since the discovery of enzyme regulation by reversible protein phosphorylation. The current excitement stems from the independent work of Krebs; and Larner's group over the period 1955-70, who discovered that the neural and hormonal control of glycogen metabolism in skeletal muscle was mediated by changes in the phosphorylation state of glycogen phosphorylase (Krebs and Fischer, 1956), phosphorylase kinase (Krebs et al., 1959) and glycogen synthase (Friedman and Larner, 1963). These three enzymes remained as the only examples of this phenomenon until late 1960s, but the situation changed rapidly following the discovery of cAMP-dependent protein kinase (Walsh et al., 1968). The past twenty years have witnessed an extraordinary and still accelerating growth in this area.

Protein phosphorylation systems consists of three primary components: a protein kinase, a protein phosphatase, and a substrate protein. The substrate protein is phosphorylated when a protein kinase transfers the terminal phosphate from ATP to the hydroxyl group of a serine or threonine or tyrosine residue of the substrate protein; the addition of charged phosphate group to the protein changes its function, presumably by changing its structure. The phosphate group is to be removed by a protein phosphatase. Protein phosphorylation is now recognized to be a major regulatory mechanism by which neural activities are controlled by external physiological stimuli (Cohen, 1982; Nestler and Greengard, 1984; Browning et al., 1985). Considerable evidence now suggests that most and possibly all of the effects of second messenger actions of cAMP, cGMP, as well as many of the second messenger actions of calcium on neuronal functions are achieved through the activation of specific cAMP or cGMP-dependent or calcium-dependent protein kinases. Vertebrate brain contains two types of cAMP-dependent protein kinases (Miyamoto et al., 1969; Walter and Greengard, 1981) and one type of cGMP-dependent protein kinase (Kuo and Greengard, 1970, Kuo and Shoji, 1982), but has multiple types of calcium-dependent protein kinases which fall into two subclasses. One subclass, activated in conjunction with calcium-binding protein calmodulin (CaM), is referred to as calcium/calmodulin-dependent protein kinase (CaM kinase). The other subclass,
activated in conjunction with phosphatidylserine and other lipids, is referred to as calcium/phospholipid-dependent protein kinase (PKC). Multiple \( \text{Ca}^{2+}/\text{calmodulin-} \) dependent protein kinases have been identified in the brain tissue of different species (Rosen and Krebs, 1981; Kikkawa et al., 1982; Kennedy et al., 1983; Nestler and Greengard, 1984; Nairn et al., 1985a, Palfrey et al., 1985; Hidaka and Ishikawa, 1992).

Although extensive investigations on protein phosphorylation systems have been carried out with the vertebrate nervous system, very little information is available for the insect nervous system. However, in the last decade, several studies have been initiated on this aspect and cyclic nucleotide-dependent protein kinases have been well characterized in insects. The cAMP-dependent protein kinases (cAMP-PKs) are ubiquitous proteins and the highly conserved structure of these kinases has been demonstrated in various species (Beebe and Corbin, 1986; Taylor et al., 1990). Properties of cAMP-dependent protein kinases of insects appear to be very similar to those of vertebrates (Foster et al., 1984; Combest and Gilbert, 1986a, 1989, Altfelder and Muller, 1991, Inoue and Yoshioka, 1997).

cAMP and cGMP-dependent protein kinases have been partially purified and characterized from various tissues and species of Arthropoda (Kuo et al., 1971). On the other hand, cAMP-PK activity has been characterized in the brain of several insects like, *Ceratitis capitata* (Haro et al., 1982), *Manduca sexta* (Combust and Gilbert, 1986a, 1989), *Apis mellifera* (Altfelder and Muller, 1991; Muller, 1997a), *Schistocerca gregaria* (Rotondo et al., 1987) and *Drosophila melanogaster* (Foster et al., 1984, Hesse and Marme, 1985, Adam and Friedrich, 1988; Inoue and Yoshioka, 1997). cAMP-PK purified from the whole body of *Bombyx mori* pupae was shown to be capable of phosphorylating rabbit skeletal muscle glycogen phosphorylase kinase and glycogen synthase, resulting in the activation and inactivation of respective enzymes (Nishiyama et al., 1975).

Genetic, biochemical and pharmacological evidences indicate an important role of cAMP-PK mediated phosphorylation in processes of learning and memory in
*Drosophila* (Aceves-Pina et al., 1983; Folkers and Spatz, 1984; Muller and Spatz, 1989; Drain et al., 1991; Muller, 1997a) and honeybee, *Apis mellifera* (Menzel et al., 1989; Sugawa et al., 1989; Muller, 1997a). Induced expression of a peptide inhibitor of cAMP-PK in transgenic *Drosophila* disrupted the ability of flies to learn an odour discrimination task (Drain et al., 1991). Immunocytochemical studies in *Drosophila* and *Apis* brain showed a 3-4 fold increase in cAMP-PK activity in mushroom bodies, when compared to other neuropile structures, in associative olfactory learning and memory (Muller, 1997a). In the sea mollusc, *Aplysia*, cAMP-PK was shown to modulate the function of ion channels during sensitization (Castellucci et al., 1982; Byrne, 1985; Byrne et al., 1991). In *Hermissenda*, microinjection of cAMP-PK into type B photoreceptors caused an increase in membrane resistance and this enhancement of light induced depolarization was found to be similar to the effects elicited by behavioural training (Alkon et al., 1983).

In addition to its role on neural function, cAMP-PK has also been reported to play essential role in insect development. Mutational analysis in *Drosophila* has shown that the adult female heterozygous for a strong and weak cAMP-PK allele fails to lay eggs and show a novel defect in oogenesis. Individuals zygotically null for cAMP-PK die as morphologically normal first-instar larva, implying that maternally encoded protein, which perdures for at least 12 h suffices for embryogenesis (Lane and Kalderon, 1993). Elevation of cAMP and the resultant activation of cAMP-PK has been reported to be a trigger for glial-to-neural cell-fate transition within the median neuroblast lineage in grasshopper (Condron and Zinn, 1995). Recent studies on *Drosophila* suggested that cAMP-PK is essential for limb development (Jiang and Struhl, 1995; Lepage et al., 1995). The prothoracic glands of *Manduca sexta* have been an advantageous model for investigating the cellular mechanisms underlying hormone-stimulated ecdysteroid secretion in insects (Rybczynski and Gilbert, 1995). The cerebral neuropeptide, prothoracicotropic hormone (PTTH) is currently thought to activate the prothoracic gland via calcium-dependent increase in cAMP synthesis, activation of cAMP-PK and protein phosphorylation (Smith et al., 1986; Gilbert et al., 1988; Smith and Gilbert, 1989; Keightley et al., 1990; Combest and Gilbert, 1992). PTTH enhances cytoplasmic cAMP content and appears to increase the amount of
cAMP bound to the regulatory subunit of cAMP-PK. This, in turn stimulates protein phosphorylation and ecdysteroid secretion by the glands and this is blocked by cAMP-PK inhibitors (Smith et al., 1996).

Among insects, cGMP-dependent protein kinase (cGMP-PK) has been well characterized in *Drosophila* and was found to be a dimer with an amino-terminal dimerization domain (Foster et al., 1996). Two *Drosophila* genes encoding products related to cGMP-PK have been isolated and the conserved position of the introns on these genes strongly suggest a common progenitor for these two genes (Kalderon and Rubin, 1989). Activity of this kinase was highest in heads of the flies and lowest in the embryos (Foster et al., 1996). cGMP-PK has also been partially purified from *Manduca* brain (Morton and Truman, 1986). Fewer biological roles have been established for cGMP. However, marked tissue variation in the concentration of cGMP-PK indicates that cGMP and its protein kinase have a more limited role in the regulation of cell function (Nestler and Greengard, 1984; Wang and Robinson, 1995). Recent experiments established a clear role for cGMP-PK in nitric oxide (NO) signalling in the nervous system of both mammals and insects (Desole et al., 1994; Bicker et al., 1996; Rodriguez-Pascual et al., 1996; Muller, 1997b; Wang and Robinson, 1997). In the honeybee, the NO/cGMP system in the antennal lobes has been implicated in the adaptive mechanisms during chemosensory processing (Muller and Hildebrandt, 1995). Experiments have also demonstrated that the action of eclosion hormone on the nervous system during the pupal ecdysis in *Manduca sexta* was mediated by cGMP (Truman, 1971) and the hormone-stimulated increase in cGMP resulted in the phosphorylation of two CNS proteins of molecular mass of 54 kDa (Morton and Truman, 1986, 1988).

Although for many years calcium has been known to be an intracellular second messenger for a wide variety of physiological processes, intracellular effectors of calcium activation remained obscure. In recent years, it has become apparent that many of the cellular actions of calcium are mediated by its binding to specific protein kinases resulting in their activation (Nestler and Greengard, 1984). Mechanism of action of calcium differs in several respects from the mechanism of action of cAMP.
and of cGMP, cAMP and cGMP act through one type of binding protein. In contrast, calcium acts through any one of the several intracellular binding proteins or in conjunction with various lipids. Furthermore, the interaction of cAMP or cGMP to its binding protein results in one effect (i.e., activation of respective protein kinase) whereas the binding of calcium to its receptor proteins results in many effects, including the activation of several enzymes (such as protein kinases). cAMP and cGMP activate only one species of respective protein kinase, whereas calcium activates several distinct species of protein kinases (Takai et al., 1979; Nairn et al., 1985b; Hidaka and Ishikawa, 1992). These observations suggested that calcium plays a more general or widespread role in cell function than do cAMP or cGMP. Multiple pathways available for calcium activation presumably enable the cell to regulate each of these pathways separately (Nestler and Greengard, 1984). Thus, regulation of protein phosphorylation is one of several mechanisms by which calcium exerts its intracellular actions. Two classes of calcium-dependent protein kinases are calcium/phosphatidylserine-dependent protein kinase (PKC) and calcium/calmodulin-dependent protein kinase (CaM kinase).

Nishizuka and colleagues (1979, 1988) discovered a new species of calcium-dependent protein kinase that is activated in conjunction with lipids. This enzyme, referred to as PKC, has been found in a wide variety of animal tissues and phyla (Kuo et al., 1980; Minakuchi et al., 1981). It phosphorylates an array of tissue specific endogenous substrate proteins (Wu et al., 1982; Nestler and Greengard, 1984; Choi et al., 1991). Total activation of PKC requires the presence of calcium and two types of lipid molecules, namely diacylglycerol (DAG) and phospholipid, such as phosphatidylserine (Kikkawa et al., 1982). Several isoforms of PKC have been identified in mammals (Nishizuka, 1988, 1995). Though in insects PKC has been characterized in Drosophila, details on isoforms are not available (Devay et al., 1989; Schaeffer et al., 1989). Three different genes of PKC have been identified in Drosophila and all are expressed in head tissue (Rosenthal et al., 1987; Schaeffer et al., 1989) and deduced amino acid sequences are quite similar to each other and to the mammalian PKC family (Schaeffer et al., 1989).
PKC's have been extensively characterized biochemically in mammals. However, the specific pathways in which they function are poorly understood and also the reason for the existence of so many different isozymes. Examples of biological processes mediated and or modulated by PKC in CNS include (1) release of neurotransmitters (Tanaka et al., 1984), (2) long-term potentiation (Malinow et al., 1988) and (3) cell growth & proliferation (Persons et al., 1988). PKC activation induces conductance changes in the photoreceptors like those seen in associative learning in the sea mollusc, *Hermissenda* (Farley and Auerbach, 1986). The *Drosophila* learning mutant, *turnip* was reported to have significant reduction in PKC activity and was also deficient in the phosphorylation of a 76 kDa membrane protein in head, which is a major endogenous substrate for PKC in wild type flies (Choi et al., 1991). Recent experiments with transgenic flies specifically inhibited for PKC resulted in the disassociation of acquisition of learning and memory from the performance of task (Kane et al., 1997). PKC has also been reported to be very essential for light adaptation in *Drosophila* photoreceptors (Hardie et al., 1993) and for outgrowth of type I and II processes of cultured mature neurons (Broughton et al., 1996a). Recently, a 86 kDa PKC specific F-actin binding substrate protein has been purified from the neural tissue of *Apis mellifera* and was shown to be closely related to myristoylated alanine-rich C kinase substrate (MARCKS) of bovine brain (Muller, 1997c).

Calmodulin (CaM) is a ubiquitous low molecular weight protein present throughout the animal kingdom. Studies on the mechanism of its action indicated that calcium binds and thereby induces structural changes in CaM. The Ca$^{2+}$/CaM complex then binds and alters the function of other cellular proteins (Carafoli, 1987; Cheung, 1980; Heizmann and Hunziker, 1990). CaM in effect, confers calcium sensitivity on other proteins which in the absence of CaM are unresponsive to calcium. Several studies done over the last two decades established that some of the second messenger actions of calcium in a variety of tissues might be achieved by the activation of Ca$^{2+}$/CaM-dependent protein kinases (Hanson and Schulman, 1992). Several Ca$^{2+}$/CaM-dependent protein kinases (CaM kinases) have been demonstrated in the neural and non-neural tissues and protein phosphorylation has been considered...
as one of the important routes by which Ca\textsuperscript{2+}/CaM signal transduction regulates cellular function. Multiple types of CaM kinases have been characterized in mammalian systems. They include CaM kinase I (Nairn and Greengard, 1987), II (Kennedy and Greengard, 1981), III (Nairn et al., 1985a), IV (Kato et al., 1992), myosin light-chain kinase (Hagiwara et al., 1989) and phosphorylase kinase (Cohen et al., 1978).

CaM kinase II is the most abundant type among the CaM kinases and has been well characterized in the neural tissue. It was first identified in rat brain as a calcium-dependent protein kinase that catalyses the phosphorylation of site 2 and 3 of Synapsin I (Kennedy and Greengard, 1981). It is highly concentrated in forebrain neurons including neurons in hippocampus, where it constitutes approximately two percent of the total protein (Erondu and Kennedy, 1985). Rat brain CaM kinase II comprises of several related isozymes (Hanson and Schulman, 1992). The rat isozymes consist of a catalytic domain, an autoregulatory domain containing a calmodulin-binding site and a C-terminal "association domain" that mediates holoenzyme formation (Bennett and Kennedy, 1987; Lin et al., 1987; Tobimatsu et al., 1988). These isozymes 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 the autophosphorylation of a threonine residue near its calmodulin binding domain which converts the enzyme to Ca\textsuperscript{2+}-independent form (Miller and Kennedy, 1986). It was shown that CaM-dependent autophosphorylation of CaM kinase I1 induces a conformational change in the region of the CaM binding domain which allows additional stabilizing interactions with CaM (Putkey and Waxham, 1996). It has been postulated that this autophosphorylation may be involved in prolonging the effects triggered by a transient calcium signal (Miller and Kennedy, 1986) and also may induce changes in the subcellular distribution of the enzyme in Aplysia and Drosophila cells (Saitoh and Schwartz, 1985; Willmund et al., 1986).

With the exception of Drosophila, there are no reports on the characterization of CaM kinase II of other insects. Adult Drosophila head contains three
species of CaM kinase II with molecular mass of 54/55, 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).

As a result of extensive studies over the last few years, it was established that CaM kinase II plays a crucial role in physiological and behavioural plasticity in both vertebrates (Malenka et al., 1989, Malinow et al., 1989, Silva et al., 1992a, b; Nayak et al., 1996; Rotenberg et al., 1996; Strack et al., 1997b) and invertebrates (Willmund et al., 1986; Griffith et al., 1993; Wang et al., 1994; Broughton et al., 1996b, Kahn and Matsumato, 1997). CaM kinase II was shown to be necessary for induction of LTP in rat hippocampus (Malenka et al., 1989; Malinow et al., 1989; Strack et al., 1997b). Also mouse strains lacking the a- subunit of this kinase, induced by the "knockout" technique, were found to be deficient in long-term potentiation (LTP) and spatial learning (Silva et al., 1992a, b; Rotenberg et al., 1996, Strack et al., 1997b). Long-term changes in the properties and subcellular distribution of CaM kinase in Drosophila heads have been demonstrated following prolonged visual adaptation (Willmund et al., 1986). Transformed strains of Drosophila expressing a transgene inhibitor of CaM kinase II have been shown to be deficient in an associative conditioning behavioural paradigm (Griffith et al., 1993; Wang et al., 1994). In contrast to the learning mutants dunce and rutabaga, which are defective in cAMP cascade, inhibition of CaM kinase II in ala transformants caused increased sprouting at larval neuromuscular junctions near the nerve entry point, rather than altering the higher order branch segments. In addition, synaptic facilitation and potentiation were altered in a manner different from that observed in the cAMP mutants (Wang et al., 1994).

One class of independent protein kinases has been found to preferentially phosphorylate acidic proteins, such as casein and phosvitin, and these enzymes are referred to as casein kinases (Hathaway and Traugh, 1982). Two types of casein kinases, known as casein kinase I (CK I) and casein kinase II (CK II), have been
identified and were found to have widespread distribution in animals (Hathaway and Traugh, 1982). CK II has been extensively studied and it was found to be a cyclic nucleotide and calcium independent protein kinase and is considered to be a tetramer with a α' β2 or α2 β2 form (Tuazon and Traugh, 1991). It is stimulated by polyamines such as spermine, spermidine and inhibited by heparin (Jacob et al., 1983; Hathaway and Traugh, 1984). In insects, CK II activity has been characterized in Drosophila (Glover et al., 1983; Birnbaum et al., 1992), and in the house cricket, Acheta domesticus (Degrelle et al., 1997). Analysis of CK II genes in Drosophila indicates a high degree of evolutionary conservation (Saxena et al., 1987).

Little is known about the functional roles of CK II in nervous tissue but few studies have speculated a role in hormonal regulation (Grande et al., 1988; Kandror et al., 1989) and processes of cell proliferation and transformation (Issenger, 1993). It is interesting to note that under in vitro conditions, several proteins were phosphorylated by CK II in the brain homogenates of Acheta domesticus (Degrelle et al., 1994) and that calmodulin and tubulin have been demonstrated to be substrates of CK II in the prothoracic glands of Manduca sexta (Song et al., 1994). More recently, it was also shown that in vivo activity of HOX protein antennapedia of Drosophila was modified because of the phosphorylation by CK II (Jaffe et al., 1997).

Protein tyrosine kinases (PTKs) are relatively recently discovered class of enzymes which specifically catalyze the phosphorylation of proteins at tyrosine residues. PTK activities have been shown to be intrinsic to the oncogene products of certain retroviruses (Hunter and Cooper, 1985) and to the receptors of several mitogenic polypeptide growth factors including epidermal growth factor (EGF), platelet derived growth factor (PDGF), insulin and fibroblast growth factor (Yarden and Ullrich, 1988). In addition, high levels of PTK activities, which are not related to either growth factor receptors or retroviral oncogene products, have also been detected in normal, uninfected cells and tissues (Swarup et al., 1983; Srivastava, 1990).
The expression of high levels of PTK activities in several differentiated and even in non-nucleated cells suggests that these kinases play important roles not only in oncogene and growth factor mediated mitogenesis, but are also involved in the regulation of differentiated and growing cells (Srivastava, 1990). Several receptor tyrosine kinases (RTKs) have been identified in *Drosophila*. The specification of the R7 photoreceptor cell in the developing eye is dependent upon the activation of *sevenless* receptor tyrosine kinase (Yamamoto, 1994; Freeman, 1996, Raabe et al., 1996). The *Drosophila* homologue of the mammalian EGF receptor has been identified as an RTK involved in many stages of development, including photoreceptor determination, wing formation, pattern formation at the anterior and posterior termini of the embryo (Doyle and Bishop, 1993, Duffy and Perrimon, 1994; Schweitzer and Shilo, 1997). A recent study suggested that bacterial lipopolysaccharide-stimulated exocytosis of non-self recognition protein from insect haemocytes depend on protein tyrosine phosphorylation (Charalambidis et al., 1995). Insulin receptor-like tyrosine kinase activity has also been reported in the prothoracic glands of *Manduca sexta* (Smith et al., 1997).

Few preliminary reports are available indicating regulation of protein kinase activity by insect hormones. Metamorphic hormone, 20-hydroxyecdysone (20E) has been shown to stimulate the *in vitro* phosphorylation of few fat body proteins of *Mamestra brassicae* (Sass, 1988) and *Sarcophaga peregrina* (Itoh et al., 1985). Injection of 20E into *Acheta domesticus* inhibits CK II activity in the brain (Degrelle et al., 1997). Juvenile hormone I (JH I) was shown to be involved in the activation of a specific Na',K - ATPase via PKC in the follicle cell membranes of *Rhodimus prolixus* (Sevala and Davey, 1989). Yamamoto et al., (1988) have reported the stimulatory role of JH on protein synthesis in male accessory glands of *Drosophila* through the activation of PKC. Eclosion hormone which triggers pupal ecdysis has been shown to stimulate the cGMP-PK activity in the CNS of *Manduca sexta* (Morton and Truman, 1986, 1988). PTTH has been shown to stimulate cAMP-PK activity in the prothoracic glands of *Manduca sexta* (Smith et al., 1996).
Scope and objectives of the present study

The analysis of the physiological functions of CaM kinase II has drawn special attention over the last few years. Three considerations justify this special interest in its role in neuronal signal transduction: (i) it is highly concentrated in brain and is localized on both sides of the synapse, where events central to neurotransmission are likely to be directly regulated, (ii) many of its substrates are involved in neuronal signalling - neurotransmitter release, synthesis and LTP, (iii) brief calcium signals activate this kinase and stimulate an autophosphorylation which allows the kinase to maintain its activated state beyond the duration of a particular calcium signal, and this would play an important role in synaptic plasticity. Although extensive investigations on CaM kinase II have been carried out with the vertebrate nervous system, little information is available for the insect nervous system. With the exception of Drosophila, there are no reports on the characterization of this kinase in other insects. The less complex insect nervous system may reveal more clearly the many roles of CaM kinase II in cellular functions. As characterization of the protein kinase is the first logical step in investigating its functional role, in the present study, an attempt was made to identify and characterize this multifunctional kinase in the CNS of the silkworm, Bombyx mori and to identify its endogenous substrate proteins. Since there are no reports on the localization of CaM kinase II protein in insects including Drosophila, the present study also makes an attempt towards this end. Finally, the present investigation also addresses the probable role of major insect hormones on general protein phosphorylation in Bombyx nervous system.