Cloning of a catalytic subunit of cAMP-dependent protein kinase from the honeybee (Apis mellifera) and its localization in the brain

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Abstract

In the honeybee the cAMP-dependent signal transduction cascade has been implicated in processes underlying learning and memory. The cAMP-dependent protein kinase (PKA) is the major mediator of cAMP action. To characterize the PKA system in the honeybee brain we cloned a homologue of a PKA catalytic subunit from the honeybee. The deduced amino acid sequence shows 80–94% identity with catalytic subunits of PKA from Drosophila melanogaster, Aplysia californica and mammals. The corresponding gene is predominantly expressed in the mushroom bodies, a structure that is involved in learning and memory processes. However, expression can also be found in the antennal and optic lobes. The level of expression varies within all three neuropiles.

Keywords: honeybee, PKA, learning, brain, mushroom body.

Introduction

The cAMP-signalling cascade is involved in learning and memory processes, as has been demonstrated by studies of several organisms ranging from insects to molluscs and mammals (for reviews see: Dubnau & Tully, 1998; Davis, 1996; Byrne & Kandel, 1996; Abel & Kandel, 1998; Brandon et al., 1997). In the honeybee the cAMP-dependent protein kinase (PKA) has been specifically implicated in learning and memory processes (Menzel, 1999). In the absence of cAMP, PKA is an inactive tetramer containing two regulatory (R) and two catalytic (C) subunits. Binding of cAMP to the regulatory subunits alters its affinity for the catalytic subunits, leading to dissociation into a dimer of regulatory subunits as well as into two active catalytic subunits. The catalytic subunits phosphorylate their substrates mediating the cellular response to alterations in cAMP level (Taylor et al., 1993).

Two structures in the honeybee brain are involved in olfactory learning and memory – the antennal lobes, which are the primary olfactory neuropils, and the mushroom bodies, which are higher order centres of multisensory integration (Menzel, 1999). In both of these structures PKA activity is induced by octopamine, the putative transmitter that mediates reward in appetitive, olfactory learning (Hammer, 1995; Müller, 1997; Hildebrandt & Müller, 1995).

Both the holoenzyme PKA II and the regulatory subunit R II, have been thoroughly characterized in biochemical and immunological studies (Altfelder & Müller, 1991; Müller, 1997). However, no PKA genes have been cloned from honeybees. Here we report the cloning of a component of the honeybee’s PKA system, namely a catalytic subunit of PKA. We present the identified cDNA sequence as well as the expression of the corresponding gene in the brain. Our results support the notion that the mushroom bodies as well as the antennal lobes are a fundamental site of PKA-based processes in the honeybee brain.

Results

Cloning of AmCO

A cDNA, named BCO, was cloned from Apis mellifera ligustica, encoding a homologue of a catalytic subunit of PKA. This partially sequenced cDNA was used as a probe to isolate a homologous cDNA of 2259 bp from Apis mellifera carnica named AmCO. The longest open reading frame of the AmCO-cDNA gives rise to a translation product of 354 amino acids. The 5’ untranslated region (UTR) contains two additional short open reading frames giving rise to thirteen and thirty amino acids. In the 3’ UTR a potential poly(A)
addition signal (AGTAAA) (Sheets et al., 1990) is located 16 bp upstream of the poly(A) tail (Fig. 1). A southern blot with genomic DNA from honeybees cut with seven different enzymes, one at a time, was hybridized with a 1 kb AmCO-fragment. This southern blot showed one band per lane (data not shown), indicating that the cloned AmCO-cDNA derives from a single copy gene.

Amino acid sequence identity with known catalytic subunits of PKA

The 354 amino acid sequence deriving from the longest open reading frame of the AmCO-cDNA shows 94% identity with the Drosophila melanogaster catalytic subunit of PKA, DCO (Foster et al., 1988; Kalderon & Rubin, 1988). Comparison with the catalytic subunits of PKA, Ca and Cb from Mus musculus (Chrivia et al., 1988; Uhler et al., 1986), and A1 and A2 from Aplysia californica (Beushausen et al., 1988) reveals amino acid identities of > 80%. Forty-three per cent of the AmCO amino acids are identical with the Saccharomyces cerevisiae catalytic subunit of PKA, TPK1 (Toda et al., 1987). All amino acid residues that are known to be relevant for the function of the catalytic subunit of PKA in mammals and yeast (Gibbs et al., 1992; Knighton et al., 1991a,b; Taylor et al., 1993; Orellana et al., 1993)
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are conserved in the derived AmCO amino acid sequence (Fig. 2) suggesting PKA phosphorylation activity of the coded protein.

Expression of the AmCO-gene in the honeybee brain

Localization of transcripts deriving from the AmCO-gene was examined by whole-mount in situ hybridization. It was performed at high stringency with a 401 bp fragment from the complete 3' non-coding region including 14 bp of the coding region of the AmCO-cDNA clone. This clone overlaps the coding region of the transcript by only 14 bp and is therefore expected to reduce the possibility of cross-hybridization to related genes. The alkaline phosphates staining reaction was allowed to proceed for 11 h. At this time strong colouration was uniformly visible in the cell bodies of the Kenyon cells within the calyces of the mushroom bodies (Fig. 3). In contrast, control (sense) probes resulted in no staining anywhere in the brain. When colourimetric detection was allowed to proceed for an additional 13 h, slight staining was visible which outlined the perimeter of the calyces. Slight staining was also just visible around the base of the antennal lobes and between the medulla and lobula within the optic lobes. At this time the controls were still devoid of staining.

In order to examine the localization of AmCO immunoreactivity in the honeybee brain a polyclonal antibody raised against recombinant DCO from D. melanogaster (Lane & Kalderon, 1993) was used due to the high degree of

sequence identity found between the DCO and the AmCO protein (see above). Specificity of this antibody for AmCO is very likely because it detects only one 40 kb band in a Western blot from honeybee brain homogenate, whose size corresponds to the calculated molecular weight of 39 kDa of the putative AmCO protein (Fiala et al., 1999).

Staining with the anti-DCO antibody showed immunoreactivity in the honeybee brain. The strongest signal was detected in the mushroom bodies, weaker signals were found in the protocerebrum, the optic lobes and the antennal lobes. Differential intensities of immunostaining were detected within the mushroom body, the antennal lobes and the optic lobes (Fig. 4).

**Mushroom bodies.** The mushroom bodies are highly ordered bilaterally symmetric neuropils that integrate multiple sensory inputs. They consist of densely packed intrinsic neurones, the so-called Kenyon cells. Interestingly, the somata of the Kenyon cells are stained differentially—strong immunoreactivity can be found in the big outward lying Kenyon cell somata, whereas the small, inwardly located Kenyon cell somata are only weakly stained. The cup-shaped input neuropils of the mushroom bodies, the so-called calyces, are dendritic arborizations of the Kenyon cells. They are subdivided into three compartments: the lip, the collar and the basal ring neuropils that differ in their sensory inputs. The lip receives input exclusively from the antennal lobes whereas the collar receives input exclusively from the compound eyes. In contrast, the basal ring receives inputs from the antennae, the compound eye and also the suboesophageal ganglia (Gronenberg, 1986; Homberg, 1984; Mobbs, 1982, 1984, 1985; Rybak & Menzel, 1993). Interestingly, these input neuropils differ in their DCO-immunoreactivity. Lip and collar are strongly stained, in comparison to the basal ring, where staining is weak. The main output neuropils of the mushroom bodies are the α- and β-lobes. In the α-lobe each calycal compartment can be allocated to a discrete band (Mobbs, 1982; Rybak, 1994). Interestingly, in the α-lobe a pattern of bands with different levels of DCO immunoreactivity can be detected. In contrast to the barely detectable DCO-immunoreactivity of the basal ring, the appropriate band in the α-lobe shows strong immunoreactivity. Nevertheless, one should take into account that it is not possible to determine whether this strong immunoreactivity is located in the intrinsic neurones of the mushroom body or in extrinsic neurones.

**Antennal lobes.** The antennal lobes are divided into spherical neuropil compartments, the glomeruli that enclose a central region of less dense neuropil. They are areas of synaptic connections between sensory neurones, interneurones and output neurones projecting to the mushroom bodies and the lateral protocerebrum. The antennal lobes show strong labelling in their neuropil whereas immunoreactivity in the flanking somata is rather poor. The staining of the glomeruli is slightly stronger than that of the central area of the neuropil.

**Optic lobes.** The three neuropil regions of the optic lobes, lobula, medulla and lamina as well as both optic chiasmata show strong immunoreactivity as compared to the fenestrated layer.

### Discussion

Here we report the cloning of a honeybee cDNA, AmCO, which encodes a homologue of the catalytic subunit of cAMP-dependent protein kinase. All amino acid residues known to be functionally relevant for the catalytic subunit of PKA in mammals and yeast (Gibbs et al., 1992; Knighton et al., 1991a,b; Taylor et al., 1993; Orellana et al., 1993) are conserved in the putative AmCO protein, suggesting a catalytic function of AmCO.

In the 5’ untranslated region of the AmCO-cDNA short upstream open reading frames (uORFs) were found. Interestingly, uORFs have also been described for transcripts...
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Coding for the PKA catalytic subunit in Drosophila melanogaster, DCO (Kalderon & Rubin, 1988) and for two splice variants of the murine Cβ gene coding for PKA catalytic subunits (Guthrie et al., 1997). In some cases, upstream open reading frames have been shown to control gene expression at the level of translation as well as mRNA turnover (Child et al., 1999; Vilela et al., 1999; Hentze, 1995; Zimmer et al., 1994). Therefore, the presence of such open reading frames in organisms as different as mammals and insects, could point to a conserved mechanism regulating expression of catalytic subunits of PKA.

Intense in situ hybridization signals of AmCO were observed in the cell bodies of the Kenyon cells. Correspondingly strong signals were detected by immunohistochemical analysis in the mushroom bodies, whereas weaker signals were observed in the lateral protocerebrum, the antennal and optic lobes. This preferential expression of AmCO in the mushroom bodies corresponds to measurements by Müller (1997) of total PKA activity in the honeybee brain. Müller showed that PKA activity in the central brain, which contains the mushroom bodies, is two to three times higher than the activity in the antennal and the optic lobes. The preferential expression of AmCO therefore reflects a predominant PKA activity in the mushroom body.

A pattern of differential AmCO gene expression can be found in the mushroom bodies. AmCO is enriched in the somata of the outward-lying big Kenyon cells, as compared to the inward-lying small Kenyon cells. In the calyces, strong immunoreactivity is found in the lip and collar, as compared to the basal ring.

The PKA holoenzyme consists of two catalytic and two regulatory subunits, therefore the ratio between regulatory and catalytic subunits in a given cell is 1 : 1 (Hofmann et al., 1977). Accordingly, regulatory subunit proteins and catalytic subunit proteins should have a similar expression pattern in the honeybee brain. Intense in situ hybridization signals of AmCO were observed in the cell bodies of the Kenyon cells. Correspondingly strong signals were detected by immunohistochemical analysis in the mushroom bodies, whereas weaker signals were observed in the lateral protocerebrum, the antennal and optic lobes. This preferential expression of AmCO in the mushroom bodies corresponds to measurements by Müller (1997) of total PKA activity in the honeybee brain. Müller showed that PKA activity in the central brain, which contains the mushroom bodies, is two to three times higher than the activity in the antennal and the optic lobes. The preferential expression of AmCO therefore reflects a predominant PKA activity in the mushroom body.

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Obvious differences between the expression patterns of AmCO and RII can be found in the optic and in the antennal lobes. In the optic lobes AmCO is strongly expressed in lobula, medulla and lamina as well as in the inner and outer chias mata. In contrast, strong RII expression can only be found in the lobula and medulla. RII expression in the lamina is very weak and is barely detectable in the chiasmata. In the antennal lobes, AmCO expression is restricted to the antennal lobe neuropil and is slightly stronger in the glomeruli than in the rest of the antennal lobe. In contrast RII immunoreactivity is concentrated in the glomeruli, and is barely detectable in the rest of the neuropil. In the glomeruli RII is differentially expressed – strong RII immunoreactivity can be found in the central area of each glomerulus whereas immunoreactivity in the rind area is low.

Thus there are several structures in both the optic and antennal lobes that show strong AmCO immunoreactivity, but either low or no RII immunoreactivity: This finding suggests that additional unknown regulatory subunits of PKA exist in these neuropils. The existence of additional regulatory subunits of PKA in the honeybee brain is likely because four RII variants have been cloned from Drosophila melanogaster and immunological studies suggest that others exist (Goodwin et al., 1991; Kaederon & Rubin, 1988; Müller, 1997). Several different regulatory subunit isoforms have also been identified in mammals (Beebe, 1994; Tasken et al., 1997). Discrete expression of a PKA catalytic subunit gene in the brain as we described it for the honeybee has been found in mice and Drosophila as well (Cadd & McKnight, 1989; Crittenden et al., 1998; Skoulakis et al., 1993). PKA catalytic subunits have been shown to be involved in neuronal plasticity and learning and memory, which is consistent with their predominant sites of expression in the mouse and fly. In vertebrates the hippocampus and the amygdala are involved in learning and memory processes (Müller et al., 1998). Transcripts coding for the PKA catalytic subunit Cβ1 can be detected in the hippocampus and transcripts coding for the PKA catalytic subunit Cβ2 show strong signals in the amygdala (Guthrie et al., 1997). Correspondingly, the inhibition of PKA activity in these brain regions leads to a reduction of hippocampal plasticity and to memory defects (Abel et al., 1997; Huang et al., 1995; Qi et al., 1996).

In Drosophila melanogaster a general involvement of PKA in associative learning was demonstrated by over-expression of a PKA inhibitor (Drain et al., 1991). The perturbation of DCO, the Drosophila melanogaster homologue of AmCO, leads to an impaired olfactory learning and medium-term memory formation (Li et al., 1995; Skoulakis et al., 1993). DCO-expression is elevated in the mushroom bodies of Drosophila melanogaster, which holds true for other components of the cAMP-signalling cascade that are involved in learning and memory in the fruit fly (Nighorn et al., 1991; Han et al., 1992; Skoulakis et al., 1993). Because the ablation of the mushroom bodies leads to defective olfactory learning in the fruit fly (DeBelle & Heisenberg, 1994, Heisenberg et al., 1985) it was supposed earlier on that the mushroom body plays a critical role in cAMP-dependent learning and memory processes. This notion was confirmed by a mushroom body specific expression of a constitutive activated stimulatory G-protein (Gαs), that leads to an impairment of olfactory learning (Connolly et al., 1996). Only recently this role of the mushroom body was specified by demonstrating that the mushroom body is minimal sufficient for cAMP-dependent short-term memory formation (Zars et al., 2000).

Comparable to the fly, in the locust and Drosophila melanogaster a general involvement of PKA in the honeybees learning and memory formation has been shown (Müller, 2000).

Also an involvement of AmCO in the honeybees learning and memory formation was demonstrated, because the reduction of AmCO amount and protein kinase A activity through antisense-oligonucleotides results in a decreased olfactory long-term memory 1–4 days after training (Fiala et al., 1999). In honeybees a classical conditioning paradigm is studied were animals learn to associate an odour with a sucrose reward. Two sites of convergent representation of odour and reward in the honeybee brain are the mushroom bodies and the antennal lobes that have been shown to be involved in learning and memory (Menzel et al., 1974; Erber et al., 1980; Hammer & Menzel, 1998). Interestingly, AmCO expression can be found in both structures. This corresponds with the notion that both structures play a role in cAMP-dependent learning and memory processes.

In the mushroom body two findings led to this conclusion: (i) the activation of PKA in cultured Kenyon cells through octopamin (Müller, 1997), and (ii) the establishment of a memory for an rewarded olfactory stimulus after replacement of the reward through an octopamin injection into the brain (Hammer & Menzel, 1998).

In the antennal lobe, a modulation of PKA activity through training has been reported (Müller, 2000) – a single olfactory conditioning trial leading to a short-term memory results in a transient increase of PKA activity that returns to basal levels 60 s after the trial. Multiple trails, in contrast, which lead to a long-term memory, prolong PKA-activity for more than 2 min in the antennal lobe. This prolonged PKA-activation is required for the induction of long-term memory (Müller, 2000). Interestingly, not only is PKA activity required for long-term memory, but also a memory trace itself is located in the antennal lobe. Joerges et al. (1997) reported spatial-temporal Ca++-signals in the glomeruli that are evoked through specific odours. The comparison of changes of these brain activities before and after olfactory conditioning, revealed a decorrelation of specific activity patterns in the glomeruli corresponding to a rewarded and unrewarded odour (Faber et al., 1999).

Molecular neurogenetics of the honeybee is just emerging (Ebert et al., 1998; Kamikouchi et al., 1998). The cloning of
AmCO together with the cloning of two receptors, a dopamine D1 receptor (AmDOP 1) and a tyrmine receptor (AmTyr1) by Biena et al. (1998, 2000), thus represents the beginning of the molecular genetic characterization of the cAMP-dependent signalling cascade in the honeybee.

The recent establishment of the antisense technique in bees, resulting in sequence-specific gene suppression (Fiala et al., 1999), makes a thorough genetic dissection of the cAMP-dependent signalling cascade in the honeybee very promising now. Cloning the components of this cascade will help us define their precise role in learning and memory formation. By identifying the sites of gene expression in the brain, it becomes possible to understand the contribution of single brain structures to these processes.

**Experimental procedures**

Cloning procedure

A honeybee (Apis mellifera ligustica) brain cDNA library (constructed from mRNA purified from the central brain, mushroom bodies, central body and deutocerebrum, using the lambda ZAP™ XR system from Stratagene) was fractionated into pools containing about 100 phages per pool. One hundred and ninety-two pools were screened by PCR using a pair of degenerated primers that derive from a consensus sequence from the catalytic subunits of PKA. Co from M. musculus (Chrivia et al., 1986; Uhler et al., 1986), DCO from Drosophila melanogaster (Foster et al., 1987; Uhler et al., 1988) and TPK 1 from S. cerevisiae (Toda et al., 1987) (Primer 1: 5′ CCA GA Y T TY GGI T TY GCI AA 3′; Primer 2: 5′ TAN CON GCN GCC ATY TGR TA 3′). For this screen 20 μl of a PCR-mixture [1× PCR-Puffer (50 m M KCl, 10 m M Tris • HCl (pH 8.3), 1 mm MgCl₂, 0.1 m M dNTPs, 0.035 U/μl Taq-Polymerase) 0.5 μM Primer 1, and 0.5 μM Primer 2] were mixed with 0.5 μl of each pool. After preheating the mixtures at 94 °C for 2 min they were denatured at 94 °C for 30 s for 40 cycles in a Perkin Elmer Cetus 9600-thermocycler. PCR-products that had the expected size of 156 bp were gel-purified, subcloned into Bluescript pSK (Stratagene) and PCR-products coding for the expected amino acid sequence were used as probes in a screen of the Apis mellifera ligustica cDNA library. The complete 3′-non-coding region including 14 bp of the coding sequence were used as probes in a screen of the Apis mellifera carnica cDNA library (constructed from mRNA purified from the central brain, mushroom bodies, central body and deutero cerebrum of Apis mellifica carnica). One clone was isolated in this screen and turned out to be almost identical with known sequences of the BCO-cDNA. The complete sequence of this cDNA-clone, named AmCO, is presented in this publication.

In situ hybridization

The complete 3′-non-coding region including 14 bp of the coding region of the presented AmCO-cDNA (401 bp) was used as a template for in vitro transcription of the in situ hybridization probes.

For in vitro transcription 250 ng of this template DNA were incubated with T3- or T7-Polymerase (10 U), placental ribonuclease inhibitor (50 U), 40 μl Tris-HCl (pH 8.25), 6 mm MgCl₂, 2 mm spermidine, 10 mm DTT, 1 mm each ATP, GTP and CTP, 0.65 mm UTP and 0.35 mm digoxigenin labelled UTP for 1 h at 37 °C. Then another 10 U of polymerase were added and incubation was allowed to proceed for an additional hour. DNA template was degraded by addition of 2 U of RNasefree DNase at 37 °C for 15 min. Free nucleotides were removed by sephadex G-50 chromatography in 50 μl Tris (pH 7.5) 500 μM EDTA, 0.1% SDS. Afterwards RNA was precipitated and resuspended in H₂O.

In situ hybridization was performed according to Christiansen et al. (1996). Bee brains were dissected from control bees and animals under a dissecting microscope in chilled phosphate buffered saline (PBS: 2 mm NaH₂PO₄, 5.8 mm Na₂HPO₄, 154 mm NaCl). Dissected brains were then placed in ice-cold fixative, 4% paraformaldehyde (PFA) in PBS for at least 1 h before the remaining bits of adhering tissue were removed. Fixation was allowed to proceed on ice overnight followed by two 10 min washes in PBTX (PBS with 0.1% triton X-100). Afterwards the fixed brains were taken through a series of methanol treatments (25%, 50%, 75%, 50%, 25% Methanol in PBTX) of 20 min each. This was followed by three washes of 10 min each in PBTX. The brains were then incubated in a 10-μg/ml solution of proteinase K in PBTX for 105 s at 20 °C and rinsed in 0.2% glutaraldehyde, 4% PFA in PBTX for 20 min on ice. Afterwards the brains were incubated at 62 °C overnight, in prehybridization mix (50% formamide, 5 × SSC, 2% Blocking Reagent (Roche Diagnostics), 0.1% triton X-100, 0.5% CHAPS, 1 mg/ml yeast RNA, 5 μM EDTA, 50 μg/ml heparin). The next day, the brains were transferred to two tubes that each contained 1 ml of fresh prehybridization mix containing approximately 50 ng of either sense or antisense probe. The brains were left to incubate at 62 °C overnight. Post-hybridization washes were all carried out at 62 °C. The first four consisted of 5 min washes in wash solution (prehybridization buffer lacking blocking powder, yeast RNA, heparin and EDTA) or wash solution diluted with 2 × SSC (2 × SSC: 3 naCl, 340 mg NaCitrate, pH 7.0) in the following ratios; 3 : 1, 1 : 1 and 1 : 3. This was followed by two 30 min washes in 2 × SSC, 0.1% CHAPS and two 30 min washes in 0.2 × SSC, 0.1% CHAPS, all at 62 °C. Finally the tissue was rinsed twice in PBTX at RT for 5 min each.

For detection the tissue was blocked with 10% sheep serum, 2% BSA in TBXT for 3 h at room temperature. One microtitre of anti-digoxigenin antibody (Roche Diagnostics, Mannheim, Germany) was diluted in 0.5 ml 10% sheep serum, 2% BSA in TBXT and pre-absorbed to 3 mg of honeybee brain powder prepared according to Harlow & Lane (1988) for 3 h at 4 °C. The brain powder was then removed by centrifugation and the supernatant diluted to 2 ml in 10% sheep serum, 2% BSA in TBXT. Brains were incubated in this antibody solution on ice overnight. Afterwards, unbound antibody was removed by five washes at 4 °C in TBXT containing 0.1% BSA and an overnight wash in the same solution. The next day the brains were washed twice in TBXT for 30 min each and three times in NTMT (100 mm NaCl, 100 mm Tris-HCl (pH 9.5), 50 mm MgCl₂, 0.1% Tween-20). Sixteen microtitrers of the phosphatase substrate mixture (Nitro Blue Tetrazolium (NBT) (18.75 mg/ml) and 5-Bromo-4-Chloro-3-Indolyphosphate (BCIP) (9.4 mg/ml in 67% DMSO) in 2 ml of NTMT) were then added to the brain. The staining reaction for the antisense samples was allowed to proceed for 11 h at 20 °C, whereas the sense samples were allowed to incubate for an additional 13 h. The stained brains were photographed and photographs were scanned and processed using the Photoshop program (Adobe, version 5.0).

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