FEBS Letters 576 (2004) 216–220 FEBS 28832

Synergistic activation of insect cAMP-dependent protein kinase A (type II) by cyclicAMP and cyclicGMP

Gérard Leboulle[1], Uli Müller*

Institut für Biologie, Freie Universität Berlin, Neurobiologie Königin-Luise-Strasse 28/30, D-14195 Berlin, Germany

Received 17 June 2004; revised 6 August 2004; accepted 17 August 2004

Available online 18 September 2004

Edited by Gianni Cesareni

Abstract The high cGMP sensitivity of cAMP-dependent protein kinase A (type II) (PKAII) from invertebrates led to the hypothesis that cGMP directly activates PKAII under physiological conditions. We tested this idea using PKAII holoenzyme purified from the honeybee brain in an assay with short stimulation times. In the presence of very low cAMP concentrations, we found a synergistic increase in PKAII activation by physiological cGMP concentrations. Cloning honeybee regulatory subunit RII and phylogenetic comparison of the two cyclic nucleotide-binding sites of RII reveal a high relation of domain A of insect RII with cGMP-binding domains of cGMP-dependent protein kinases.

Keywords: Insect; Protein kinase A; Regulatory subunit; Cyclic AMP; Cyclic cGMP; Nucleotide-binding site

1. Introduction

The cAMP signal transduction system plays a central role in many cellular processes ranging from proliferation, differentiation and growth to synaptic plasticity in the nervous system [1]. Cyclic AMP-dependent protein kinases (PKA), the major mediators of cAMP actions, are tetrameric enzymes (R_2C_2) that consist of two regulatory subunits (R) and two catalytic subunits (C). In mammals, four genes encode two classes of R subunits (RIα, β and RIIα, β) and three genes encode the catalytic subunits (Cα, β, γ) [2]. Analysis of catalytic and regulatory subunits (R) from numerous species, including invertebrates and protozoans, demonstrates the high conservation of PKA subunits throughout the animal kingdom [1,3].

Dissociation of the C subunits and thus activation of PKA is mediated by cAMP-binding to two nucleotide-binding domains, domains A and B, which act as fast and slow activating domains, respectively [4,5]. Within each domain, the phosphate-binding cassette (PBC), a short sequence of about 20 amino acids, interacts with the cyclic nucleotide [6,7]. The PBCs of the R subunits are highly conserved and differ in only a few amino acids from the PBC of the cGMP-dependent protein kinases (PKG) [2]. Although a few amino acids in the PBCs are very critical for nucleotide specificity, it is the PBC in total that determines the individual nucleotide specificity of a given R subunit [8–11]. While activation of mammalian PKA requires a 50- to 100-fold higher concentration of cGMP compared to cAMP [10], the discrimination between cAMP and cGMP is not so clear in invertebrates. In some cases, PKA activation requires much lower cGMP concentrations [12–14] and in *Trypanosoma brucei*, PKA is activated only by cGMP [15]. In this study, we test the hypothesis whether cAMP and cGMP are both involved in direct PKA activation as proposed in recent in vivo studies in honeybees [16–18].

2. Materials and methods

2.1. Materials

Apis mellifera were taken from a regular hive. [γ-^{32}P]ATP (5000 Ci/mmol) was purchased from NEN Life Science Products (Brussels, Belgium). Phosphatase inhibitor I1 was purified from bovine brain according to the procedure described for skeletal muscle [19]. PKA inhibitor peptide, PKI (6–22) and all other chemicals were obtained from Sigma (Deisenhofen, Germany).

2.2. Purification of the PKAII holoenzyme

PKAII holoenzyme was purified according to the methods of [12,20]. Brains of 100 bees (~120 mg) were dissected and homogenized in 5 ml buffer-A (50 mM Tris–HCl, pH 7.8, 1 mM EDTA, 1 mM EGTA and 10 mM 2-mercaptoethanol). After centrifugation (50 000 × *g*; 30 min), the supernatant was applied to a DEAE-Sepharose column (2 ml, equilibrated in buffer-A). Fractions between 0.18 and 0.23 M NaCl, containing PKAII activity, were dialyzed against buffer-B (10 mM sodium phosphate-buffer, pH 6.8, 1 mM EDTA, 1 mM EGTA and 10 mM 2-mercaptoethanol) and passed over a CM-Sephadex column (5 ml) equilibrated in buffer-B. The flow-through fraction, containing PKAII holoenzyme, was slowly (2 ml/h) passed over an adenosine 3′:5′-cyclic monophosphate agarose (C-8) affinity column (0.5 ml). After washing with 15 ml of 2 M NaCl in buffer-B, followed by 15 ml buffer-B, the RII subunit was eluted by 8 M urea in buffer-B. The fractions (0.2 ml) containing RII were dialyzed in buffer-B containing 0.1 M NaCl. The flow-through fraction of the affinity column, containing the catalytic subunit, was dialyzed against buffer-B and passed over a CM-Sephadex column (0.5 ml). After washing with buffer-B, the C subunit was eluted with 0.5 M NaCl. The fractions (0.2 ml) containing catalytic activity were dialyzed against buffer-A containing 0.1 M NaCl. Fractions with purified RII and C subunits were pooled and the reconstituted PKAII holoenzyme was separated from free subunits

* Corresponding author. Present address: Universität des Saarlandes, Naturwissenschaftlich-Technische Fakultät III, (FR 8.3) Biowissenschaften, Zoologie/Physiologie, Postfach 151150, D-66041 Saarbrücken, Germany. Fax: +49-681-3026652.
E-mail addresses: gerleb@zedat.fu-berlin.de (G. Leboulle), uli.mueller@mx.uni-saarland.de (U. Müller).

[1] Fax: +49-30-83855455.

Abbreviations: C, catalytic subunit; R, regulatory subunit; PBC, phosphate-binding cassette; PKA, cAMP-dependent protein kinase; PKG, cGMP-dependent protein kinase

0014-5793/$22.00 © 2004 Published by Elsevier B.V. on behalf of the Federation of European Biochemical Societies.
doi:10.1016/j.febslet.2004.08.079

using a Sephacel S300 column (equilibrated in buffer-A containing 0.1 M NaCl) and used immediately.

2.3. Determining PKA activity

PKA activation by a short (30 s) cyclic nucleotide stimulation was measured by using phosphatase inhibitor 1 (I1), a specific PKA substrate [21]. For phosphorylation, samples of the purified PKAII holoenzyme (10 μl) were mixed with 10 μl phosphorylation buffer (50 mM Tris-buffer, pH 7.5, 0.1 M NaCl, 30 μM ATP, 1 μCi [γ-^{32}P]ATP (5000 Ci/mmol), 20 mM $MgCl_2$, 10 mM 2-mercaptoethanol and 1 μg I1 boiled for 2 min prior to use). The phosphorylation mixture contains cAMP and/or cGMP at concentrations as indicated in Section 3. After incubation for 30 s at 20 °C, the reactions were terminated by adding 5 μl of SDS-sample buffer (0.5 M Tris-buffer, pH 6.8, containing 5% sodium dodecyl sulfate (SDS), 5% mercaptoethanol, 20% glycerol, and 0.1% bromophenol blue). SDS–PAGE and determination of ^{32}P-incorporation into I1 were quantified as described in [18].

2.4. Cloning of the regulatory subunit RII of honeybee PKA

The AmPKA-R2 mRNA sequence (Accession No. AJ698737) was identified by using the tBLASTn and BLASTn algorithms (default parameters) to search a honeybee EST library [22] and the honeybee genome (www.hgsc.bcm.tmc.edu/projects/honeybee/). Specific primers, hybridizing in the 5′ and 3′ untranslated regions, were synthesized from these sequences.

Five honeybee workers (*Apis mellifera carnica*) were collected from the hive and immobilized on ice. After dissection, brains were immediately crushed, using a cell homogenizer, in a 1.5 ml tube containing Trizol reagent and total RNA was extracted according to the manufacturer's instructions (Invitrogen, Karlsruhe, Germany). Two micrograms of RNA was reverse-transcribed by using an oligo-d$(T)_{18}$ primer and the Revertaid H minus first-strand cDNA synthesis kit (MBI Fermentas, St.Leon-Rot, Germany). This cDNA was used as template in a PCR, including 1 unit of Gentherm polymerase (Rapidozym, Berlin, Germany), dNTPs (250 μM each dNTPs), the direct primer 5′-ATTTGCCTATTATTTGCCAGTTA-3′ (1 μM), the reverse primer 5′-AAAACACGCGGATGAGATTC-3′ (1 μM) and the PCR buffer (16 mM $(NH_4)_2SO_4$, 67 mM Tris–HCl (pH 8.8), 1.5 mM $MgCl_2$ and 0.01% Tween 20). The PCR protocol consisted of 25 cycles of 30 s at 95 °C, 30 s at 62 °C, and 1 min at 72 °C, followed by a 10 min incubation at 72 °C. The amplified cDNA was ligated into the pCR4-TOPO cloning vector and TOP10 *Escherichia coli* cells were transformed with the ligation product (Invitrogen, Karlsruhe, Germany). Several clones obtained from different RT-PCR, were sequenced and the identified sequence was deposited at GenBank/EMBL.

2.5. Computational analysis

The phylogenetic trees were realized from sequence alignment of AmPKA-R2, human PKA-R1α (NM-002734) and β (NM-002735), PKA-R2α (NM-004157) and β (NM-002736), *Mus musculus* PKA-R2α (NM-008924) and β (NM-011158), *Drosophila melanogaster* PKA-R2 (NM-176117) and PKA-R1 (P16905), *Caenorhabditis elegans* PKA-R (NM-076598), *Aplysia californica* PKA-R1 (CAA44246) and PKA-R2 (AY387673), *Anopheles gambiae* PKA-R2 (XM-315029), *Apis mellifera* PKG (AF469010), *Drosophila melanogaster* PKG (AAA28459), *Bombyx mori* PKG (AF465600), human PKG1 (NM006258) and PKG2 (NM006259), *Mus musculus* PKG1 (NM011160) and PKG2 (NM-008926) by the ClustalW alignment method with the Gonnet series protein weight matrix (default parameters) for the whole sequence alignment in Fig. 2 and by the Jotun Hein method with the structural protein weight matrix (Default parameters) for the PBC alignments in Fig. 3 (Megalign software – DNASTAR, Madison, WI, USA).

3. Results and discussion

3.1. Synergistic activation of purified PKAII holoenzyme by cAMP and cGMP

Previous in vivo studies on the interaction between the NO/cGMP-pathway and PKA activation in honeybees led to the conclusion that PKAII activity is directly modulated by both cAMP and cGMP [16–18]. To test this hypothesis, we used PKAII holoenzyme purified from bee brain and purified phosphatase inhibitor-1 (I-1) as substrate to exclude enzyme activities other than PKA [16–18,21]. The separate purification of the C subunits and the RII subunits followed by a reconstitution of the RII_2C_2 holoenzyme allows the verification of their purity at various steps. By crosslinking potentially bound proteins with the subunits or the holoenzyme, followed by SDS–PAGE separation and sensitive silver staining techniques or Western blot analysis with antibodies against the subunits, we excluded the possibility that other proteins are bound to one of the subunits or the reconstituted holoenzyme. However, due to the resolution of the separation techniques (ionic strength and molecular weight), a contamination with small peptides cannot be totally excluded.

In order to keep close to physiological activation dynamics, we used a short cyclic nucleotide stimulation of 30 s. Under these conditions, half-maximal activation of PKAII requires 0.1 μM cAMP or 3 μM cGMP (Fig. 1A). Both the cAMP and the cGMP activation are totally abolished by a specific PKA inhibitor peptide (Fig. 1B), demonstrating that the measured activity is due to the action of the catalytic subunit.

However, with respect to the physiological situation in living cells, the isolated stimulation by either cAMP or cGMP used in most biochemical studies is not well suited to uncover potential effects of the coincident presence of cAMP and cGMP on PKA activation (or any other targets of the cyclic nucleotides). The latter is a well-known problem of physiological studies on intact cells or intact animals, where a clear-cut discrimination between cGMP and/or cAMP action on their targets is often not possible [23]. Especially, the cGMP production via the soluble guanylyl cyclase (sGC), which is not coupled to a distinct receptor and is directly activated by the diffusible nitric oxide (NO), led to the hypothesis that cGMP, at low cAMP concentrations, may directly activate PKAII [16–18].

Based on this hypothesis, we tested the effect of cGMP on PKAII holoenzyme activation in the presence of low concentrations of cAMP (Fig. 1C). CyclicGMP at low physiological concentrations is unable to activate PKAII. However, in the presence of cAMP in the nanomolar range (25 or 33 nM) that alone activates PKAII in the range of 4–10%, these low cGMP concentrations lead to a synergistic and significant additional PKAII activation. Whereas, for example, 250 nM cGMP alone activates PKAII less than 1%, it leads to an additional 12% PKAII activation in presence of 25 nM cAMP, which alone activates PKAII about 4%. Although only in the range 10–15% of maximal PKAII activation, the observed synergistic effect is sufficient to explain the in vivo PKAII activation during associative and non-associative learning in the honeybee, which has been proposed to be mediated by direct cGMP action on PKAII [16–18]. Moreover, the synergistic effect of cGMP on PKAII activity at low cAMP concentrations points to a difference in the properties of the two cAMP-binding domains of the regulatory subunit. Our findings are consistent with the idea that the cAMP-binding domain B (slow-binding domain), which is occupied first, is sensitive to cAMP but not to cGMP. Thus, the initial binding of cAMP to domain B is required to expose binding domain A (fast binding domain), which seems to be more sensitive to cGMP as compared to domain B. This, together with the frequently reported difference in cAMP versus cGMP selectivity of invertebrate PKAII and mammalian PKAII, prompted us to analyze the regulatory subunit and especially the cAMP-binding domains in more detail.

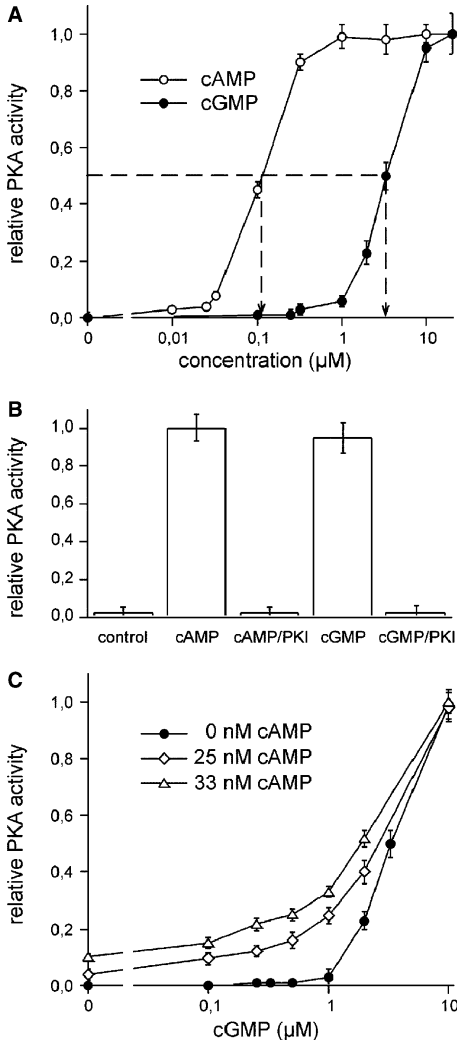

Fig. 1. Cyclic nucleotide-dependent activation of cAMP-dependent protein kinase A (PKAII) from honeybees. Activity of PKAII-holoenzyme purified from honeybee brains was determined using phosphatase inhibitor I as substrate. Kinase activation by cAMP and/or cGMP was terminated after 30 s to keep stimulation within a physiological time range. The ^{32}P-incorporation into the substrate was normalized with respect to the maximal value (20 μM cAMP). (A) Activation of PKAII holoenzyme by either cAMP or cGMP at concentrations as indicated. The arrows indicate concentrations required for half-maximal activation. Each value presents the mean (±S.E.M.) of at least 8 measurements. (B) Addition of the specific PKA inhibitor peptide PKI-(6-22) amide completely abolishes the cAMP (10 μM) or cGMP (10 μM) stimulated kinase activation. Each column presents the mean (±S.E.M.) of 10 measurements. (C) Effect of cGMP on PKAII activity in the absence and presence of cAMP. Activation of PKAII by cGMP (0.1–3.3 μM) in the presence of cAMP significantly differs from activation by cGMP alone. Each point presents the mean (±S.E.M.) of at least 12 measurements (*t* test: $P < 0.001$).

3.2. Molecular analysis of the cAMP-binding domains of the PKAII regulatory subunit

As expected, the amino acid sequence of AmPKA-R2 reveals all general structural features of the PKA-R2 gene family, including dimerization site, catalytic subunit binding site with the autophosphorylation site, and the two cAMP-binding domains comprising the PBC [1,20].

Phylogenetic analysis of several PKA-Rs and PKGs show that the compared proteins are clustered in separate groups that are consistent with accepted phylogenetic relationships (Fig. 2). AmPKA-R2 shows the highest similarity with PKA-R2 from *Anopheles gambiae* (71.1% amino acids identity) and *Drosophila melanogaster* (61.1% amino acids identity).

Since the biochemical data suggest a difference between the cAMP-binding domains, the PBC domains A and B from AmPKA-R2 were compared with the corresponding sequences of PKA regulatory subunits and PKGs of different species. A phylogenetic tree of the PBC domains A and B was realized with the Jotun Hein method by using the structural protein weight matrix of the DNASTAR software. This alignment method was preferred because it gives higher scores to residues that are chemically and spatially similar. Analysis using the ClustalW slow-accurate method with the Gonnet protein weight matrix revealed similar results as compared to the Jotun Hein analysis (data not shown).

The analysis of the different PBC domain A shows that the first branching of the tree separates PKGs and PKA-Rs (Fig. 3A). In the PKA-R branching, there is an important divergence between the different PBCs. Indeed, the *Drosophila* PBC is diverging early in the PKA-R branching, followed by the *Anopheles* and the honeybee PBCs (Fig. 3A). The next branching separates the *Aplysia* sequence from a group comprising the mammalian PKA-R2 and all analyzed PKA-R1 domains that are the most distant from the root of the tree.

In the PBCs domain B tree, the first branching is also separating PKGs and PKA-Rs (Fig. 3B). In the PKA-R branching, the different domains are clustered in sub-groups that are more distant from the root of the tree and more nucleotide substitutions are used to construct this tree in comparison with the domain A tree. In the PKA-R group, the first branching separates PKA-R1 from PKA-R2 subunits. Interestingly, the different sub-groups composing the PKA-R group are not totally consistent with accepted phylogenetic relationships. For example, the *Drosophila* PKA-R2 sequence is localized in the mammalian PKA-R2 sub-group rather than in the insects sub-group. It is unlikely that the *Drosophila* PBC domain B is more related to mammalian PBC domain B. Indeed, the *Drosophila* PBC is presenting 90.5% identity to the *Anopheles* PBC, 81% to the honeybee and the human PKA-R2β PBCs, and 85.7% identity to the human PKA-R2α domain. This classification is probably due to the fact that the PBC domain B of PKA-Rs is highly conserved and composed of short stretch of sequence.

These phylogenetic tree analyses indicate that the PBC domain A of insect PKA-R2s are more related to a common ancestor of PKA-R2 and PKGs PBCs than the PBC domain A of other PKA-R subunits. On the contrast, the domain B analysis shows that all the PKA-R domains are clustered in sub-groups much more distant from the root of the tree, suggesting that they are less related to PKGs PBCs.

Finally, the different sequences were manually annotated by focusing on conserved amino acids within the different PBCs, because these are likely to represent residues that serve important functions (Fig. 3C and D). This annotation shows that the sequences of PKG and PKA-R2 PBCs A and B are highly conserved and that almost all amino acids interacting with the cyclic nucleotide are identical, except at position 15, which is characterized by an alanine in PKA-Rs and by a threonine or a serine in PKGs (Fig. 3C and D). Other amino acids composing the PBCs domains A and B are particular to all PKA-Rs (Fig. 3C and D – light gray boxes) or to all PKGs (Fig. 3C and

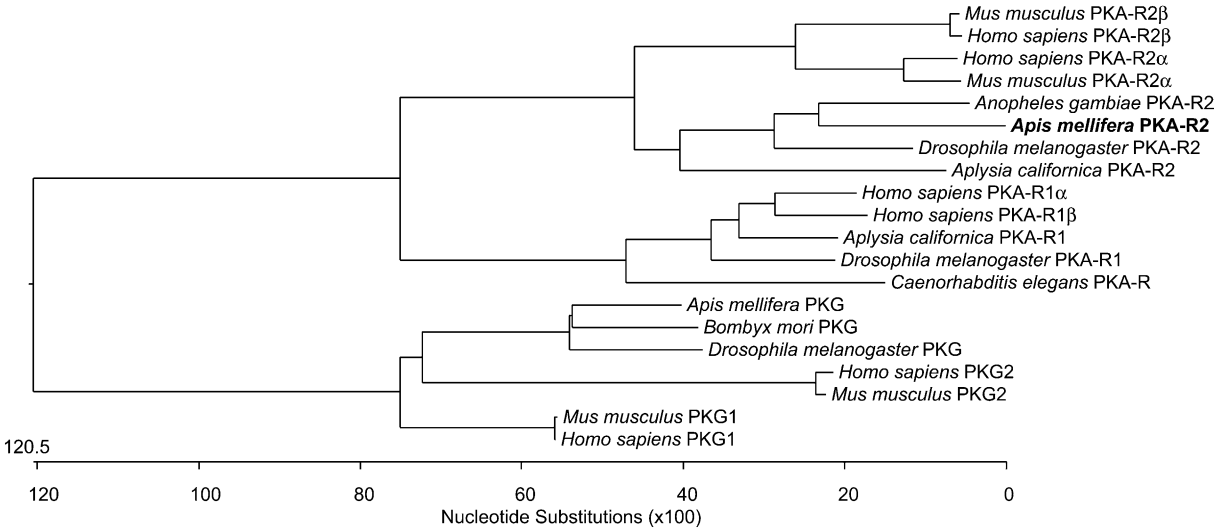

Fig. 2. Phylogenetic analysis of PKA-R subunits and PKGs. The predicted amino acid sequence of AmPKA-R2 was compared with different proteins. The distances between the branches correspond to sequence divergence. The scale indicates the nucleotide substitution.

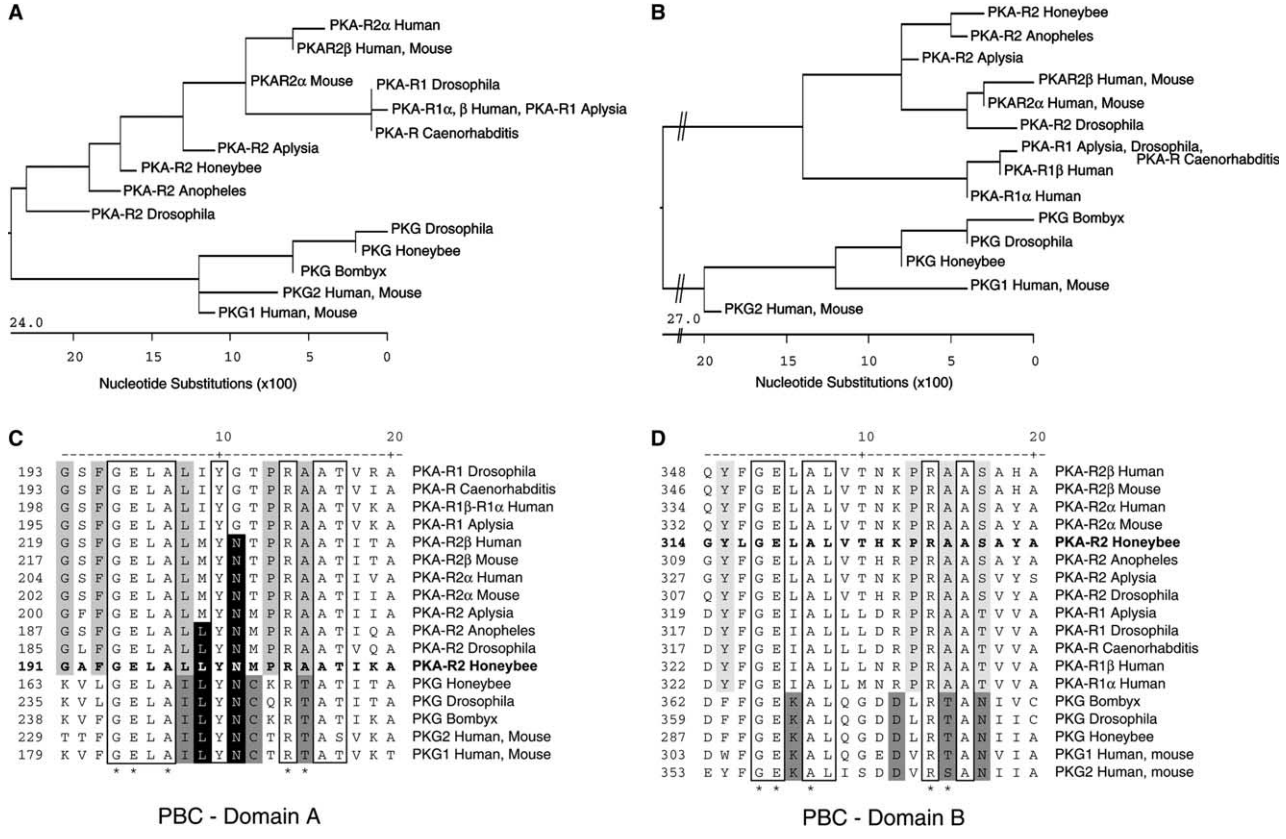

Fig. 3. Analysis of the PBCs of PKA-R subunits and PKG. The phylogenetic tree of PBCs domains A (A) and B (B) compared the predicted amino acid sequence of AmPKA-R2 with different proteins. The distance between the branches corresponds to sequence divergence. The scale indicates the nucleotide substitution. The amino acids sequences of PBC domains A (C) and B (D) have been manually notated. The boxes indicate amino acids, which are identical in all the sequences; the asterisks mark the amino acids directly interacting with the cyclic nucleotide. The light and dark gray boxes show the amino acids that are identical in all PKA-R or all PKGs, respectively. The black boxes show PKGs conserved amino acids that are also present in PKA-R subunits. The position of the first amino acid of PBC domains is indicated on the left and the relative position of each amino acid in the PBC is indicated at the top of the alignment.

D – dark gray boxes). It is probable that these conserved amino acids are also important in determining the PBC specificity either for cAMP or for cGMP. Interestingly, the analysis of the domain A showed that a conserved leucine in position 9 of all PKGs is also found in the PBC domain A of insects PKA-R2 and that the conserved asparagine in position 15 of

all PKGs is also found in all PKA-R2 domain A (Fig. 3C – black boxes). Such similarities between PKGs and PKA-Rs are not found in the PBC domain B (Fig. 3D).

In conclusion, these results indicate that the PBC domain A of insects PKA-R2 is an atypical cyclic nucleotide-binding domain. The phylogenetic and the sequence analyses demonstrate that it is a cAMP-binding site, but it also shows similarities to the PBC domain A of PKGs that could explain the synergistic action of cAMP and cGMP on PKA activation in the honeybee. However, only site directed mutagenesis experiments could validate these predictions.

Although we have only demonstrated a synergistic activation of PKAII by cAMP and cGMP in honeybees, the sequence comparison points to a general feature of insect PKAIIs with their corresponding consequences regarding the interaction between the cGMP- and the cAMP-regulated signaling cascades.

Acknowledgements: We thank the BCM-HGSC (www.hgsc.bcm.tmc.edu/projects/honeybee/) for making available the honeybee genome.

References

[1] Taylor, S.S., Buechler, J.A. and Yonemoto, W. (1990) Annu. Rev. Biochem. 59, 971–1005.
[2] Francis, S.H. and Corbin, J.D. (1999) Critical Rev. Clin. Lab. 36, 275–328.
[3] Canaves, J.M. and Taylor, S.S. (2002) J. Mol. Evol. 54, 17–29.
[4] Shabb, J.B. and Corbin, J.D. (1992) J. Biol. Chem. 267, 5723–5726.
[5] Woodford, T.A., Correll, L.A., McKnight, G.S. and Corbin, J.D. (1989) J. Biol. Chem. 264, 13321–13328.
[6] Su, Y., Dostmann, W.R., Herberg, F.W., Durick, K., Xuong, N.H., Ten Eyck, L., Taylor, S.S. and Varughese, K.I. (1995) Science 269, 807–813.
[7] Diller, T.C., Xuong, N.H. and Taylor, S.S. (2000) Protein Expr. Purif. 20, 357–364.
[8] Shabb, J.B., Ng, L. and Corbin, J.D. (1990) J. Biol. Chem. 265, 16031–16034.
[9] Shabb, J.B., Buzzeo, B.D., Ng, L. and Corbin, J.D. (1991) J. Biol. Chem. 266, 24320–24326.
[10] Reed, R.B., Sandberg, M., Jahnsen, T., Lohmann, S.M., Francis, S.H. and Corbin, J.D. (1996) J. Biol. Chem. 271, 17570–17575.
[11] Taylor, M.K. and Uhler, M.D. (2000) J. Biol. Chem. 275, 28053–28062.
[12] Altfelder, K. and Müller, U. (1991) Insect Biochem 21, 487–494.
[13] Frajnt, M.., Cytrynska, M. and Jakubowicz, T. (2003) Acta Biochim. Pol. 50, 1111–1118.
[14] Locatelli, F., LaFourcade, C., Maldonado, H. and Romano, A. (2001) J. Comp. Physiol. [B] 171, 33–40.
[15] Shalaby, T., Liniger, M. and Seebeck, T. (2001) Eur. J. Biochem. 268, 3206–6197.
[16] Müller, U. (2000) Neuron 27, 159–168.
[17] Müller, U. and Hildebrandt, H. (1995) Eur. J. Neurosci. 7, 2240–2248.
[18] Müller, U. and Hildebrandt, H. (2002) J. Neurosci. 22, 8739–8747.
[19] Huang, F.L. and Glinsmann, W.H. (1976) Eur. J. Biochem. 70, 419–426.
[20] Foster, J.L., Guttman, J.J., Hall, L.M. and Rosen, O.M. (1984) J. Biol. Chem. 259, 13049–13055.
[21] Hildebrandt, H. and Müller, U. (1995) J. Neurobiol. 27, 44–50.
[22] Whitfield, C.W., Band, M.R., Bonaldo, M.F., Kumar, C.G., Liu, L., Pardinas, J.R., Robertson, H.M., Soares, M.B. and Robinson, G.E. (2002) Genome Res. 12, 555–566.
[23] Zufall, F. and Munger, S.D. (2001) Trends Neurosci. 24, 191–193.