J Comp Physiol A (2003) 189: 427–434
DOI 10.1007/s00359-003-0419-x

ORIGINAL PAPER

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Activity of protein kinase A and gustatory responsiveness in the honey bee (*Apis mellifera* L.)

Received: 11 December 2002 / Revised: 4 February 2003 / Accepted: 28 March 2003 / Published online: 25 April 2003

Abstract The cAMP-dependent protein kinase A is involved in the induction of long-term memory and habituation in the bee. Gustatory responsiveness correlates strongly with associative and non-associative learning in bees. We tested whether protein kinase A activity in the antennal lobes correlates with gustatory responsiveness. Thirty minutes after feeding, bees with high gustatory responsiveness had a significantly higher protein kinase A activity than bees with low responsiveness. Ninety minutues after feeding, when gustatory responsiveness had increased in initially unresponsive bees, no changes in protein kinase A activity were found. We also tested time-dependent effects of protein kinase A activator and protein kinase A inhibitor on gustatory responsiveness. Injection of the protein kinase A activator adenosine 3'5'-cyclic monophosphate 8-bromo-sodium salt or of the protein kinase A inhibitor KT 5720 did not affect gustatory responsiveness within the first 4 h after treatment. Feeding of adenosine 3'5'-cyclic monophosphate 8-bromo-sodium salt over 4 days increased gustatory responsiveness in newly emerged bees and adult foragers. These results enable us to distinguish between two different forms of gustatory responsiveness: *basal* and *transient* gustatory responsiveness. Basal gustatory responsiveness correlates with protein kinase A activity and can only be modulated in the range of several days. Transient gustatory responsiveness appears to be independent of protein kinase A activity and can be modulated in the range of minutes to hours.

Keywords cAMP system · Gustatory responsiveness · Honey bee · PER · Protein kinase A

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Abbreviations 8-Br-cAMP adenosine 3'5'-cyclic monophosphate 8-bromo-sodium salt · GRS gustatory response score · PER proboscis extension response · PKA protein kinase A · PKG protein kinase G

Introduction

In the last few years, there has been increasing evidence that changes in synaptic connectivity are the neural substrate for behavioural plasticity induced by associative and non-associative learning (Milner et al. 1998). In this context, different protein kinases have been shown to play important roles as mediators of phosphorylation processes. Protein kinase A (PKA), which is regulated by cAMP, has been shown to be involved in behavioural plasticity of divers animal phyla. In the sea-slug, *Aplysia californica*, PKA mediates the sensitisation of the gill-withdrawal reflex (for a review see Milner et al. 1998). Mutants of the fruit fly, *Drosophila melanogaster*, have demonstrated an important role of PKA in associative and non-associative learning and memory formation (for a review see Dubnau and Tully 1998). In vertebrates, a connection has been shown between PKA activity and long-term potentiation in the hippocampus (Abel et al. 1997). In the honey bee, PKA, is involved in the induction of long-term memory during associative learning (Fiala et al. 1999; Müller 2000) and in the habituation of the proboscis extension response (Müller and Hildebrandt 2002).

The perception of sucrose stimuli is essential for non-associative and associative learning in the bee. Non-associative habituation is usually tested by applying sucrose stimuli to the antenna, which elicits the proboscis extension response (PER). After a number of stimulations the PER is reduced or vanishes completely (Menzel et al. 1991; Braun and Bicker 1992; Müller and Hildebrandt 2002). Under free-flying conditions, bees learn to associate sensory cues such as colours, odours,

visual patterns or landmarks with a sucrose reward (Menzel and Müller 1996). In laboratory experiments on classical olfactory conditioning or tactile conditioning, sucrose serves as the unconditioned stimulus and as the reward (Takeda 1961; Bitterman et al. 1983; Erber et al. 1998; Scheiner et al. 2001a, 2003). In operant conditioning paradigms in the laboratory, sucrose also represents the reward signal reinforcing antennal scanning movements or the activity of a single antennal muscle (Kisch and Erber 1999; Erber et al. 2000).

The individual responsiveness to water and sucrose stimuli is an important determinant of associative and non-associative learning in the bee. It can be quantified by measuring the occurrence of PER when the antennae of a bee are stimulated with water and different sucrose concentrations (Page et al. 1998; Scheiner et al. 2001a, 2001b, 2003). This gustatory responsiveness was shown to correlate with acquisition and long-term memory in associative olfactory and tactile learning. It can explain learning differences between bees with different foraging roles, bees of different genotypes and bees of different ages (Scheiner et al. 1999, 2001a, 2001b). Gustatory responsiveness also correlates with habituation of PER (Scheiner 2001) and phototactic behaviour (J. Erber, personal observation). Thus, gustatory responsiveness is an excellent indicator of the behavioural state of a bee.

Interestingly, there are striking similarities between the modulation of PKA activity in the honey bee and the modulation of gustatory responsiveness. The injection of octopamine, which leads to an increase in gustatory responsiveness in the range of minutes to hours (Scheiner et al. 2002), also increases the PKA activity in the antennal lobes (Hildebrandt and Müller 1995), although on a much shorter time scale. Stimulation of the antennae with sucrose can increase behavioural and neuronal responsiveness for gustatory stimuli and other stimulus modalities (Pribbenow and Erber 1996; Scheiner 2001). In the antennal lobes, the first olfactory neuropil of the bee brain, a rapid increase in PKA activity which lasts up to 3 s has been demonstrated (Hildebrandt and Müller 1995). So far, it has not been determined whether PKA is part of the pathway modulating gustatory responsiveness. Because individual gustatory responsiveness can predict associative learning and many other behaviours in the honey bee, it is essential to analyse the relationship between PKA and gustatory responsiveness. The results which we gain from the honey bee study will help clarify the role of PKA in other model systems such as *Drosophila*, *Aplysia* and vertebrates. The activity of PKA was measured in the antennal lobes of bees with different gustatory responsiveness. The PKA activator adenosine 3'5'-cyclic monophosphate 8-bromo-sodium salt (8-Br-cAMP) and the PKA inhibitor KT 5720 were tested for their modulatory effects on gustatory responsiveness.

Materials and methods

PKA activity in the antennal lobes of foragers

For this experiment, returning non-pollen foragers were caught individually at the hive entrance, cooled until they showed first signs of immobility in a refrigerator maintained at 4°C and mounted in metal tubes as described earlier (Erber et al. 1998). Bees were fed to satiation with 30% sucrose solution. Between 20 and 25 min after feeding, gustatory responsiveness was measured using the proboscis extension response as described earlier (Page et al. 1998; Scheiner et al. 1999, 2001a, 2001b). Bees were tested for their PER by stimulating them with water, 0.1% sucrose, 1% sucrose, 10% sucrose and 30% sucrose. In this experiment, only four sucrose concentrations were tested for two reasons. Firstly, we wanted to measure PKA activity 30 min after feeding, which did not leave us enough time to test six different sucrose concentrations starting 20 min after feeding. Secondly, we only measured PKA activity of bees with minimal or maximal responsiveness. To find bees with minimal or maximal responsiveness it was not necessary to test all six sucrose concentrations.

In all of the following experiments six sucrose concentrations were used. The total number of proboscis extensions of a bee to the different sucrose concentrations constitutes its gustatory response score (GRS), which is an excellent measure of its gustatory responsiveness (Scheiner et al. 1999, 2001a, 2001b). In the first experiment, the GRS of an individual could vary between 0 (no PER to any of the stimuli tested) and 5 (PER to all of the stimuli tested). In all later experiments, the GRS could vary between 0 and 7. Individuals were selected for two main experimental groups according to their GRS: (1) bees with very high gustatory responsiveness (GRS = 5; these bees responded to water and all sucrose concentrations), and (2) bees with very low gustatory responsiveness [GRS = 1 or 0; these bees responded only to 30% sucrose or did not respond at all; typically those bees only respond to stimulation with honey or 60% sucrose (R. Scheiner, personal observation)].

Bees were then separated into six subgroups which were tested differently (see Table 1). In subgroup 1 (very high responsiveness) and in subgroup 4 (very low responsiveness) PKA activity was measured in the antennal lobes 5 min after measurement of GRS, i.e. 30 min after feeding. In subgroup 2 (very high responsiveness) and subgroup 5 (very low responsiveness) PKA activity was measured in the antennal lobes 90 min after feeding. In subgroup 3

Table 1 Experimental subgroups for protein kinase A (PKA) activity measurements (*GRS* gustatory response score)

Subgroup	Gustatory responsiveness	Time of experiments after feeding (min)	
		Time of GRS measurement	Measurement of PKA activity
1	Very high (GRS = 5)	20–25 min	30 min
2	Very high (GRS = 5)	20–25 min	90 min
3	Very high (GRS = 5)	20–25 and 85–90 min	
4	Very low (GRS ≤ 1)	20–25 min	30 min
5	Very low (GRS ≤ 1)	20–25 min	90 min
6	Very low (GRS ≤ 1)	20–25 and 85–90 min	

(very high responsiveness) and subgroup 6 (very low responsiveness) gustatory responsiveness was tested at 20 and 90 min after feeding.

In order to measure PKA activity the heads of the bees were ducked in liquid N_2, cut off, and stored in liquid N_2 until they were subjected to incomplete lyophylisation (3.5 h at –20°C, 0.05 mbar, Hildebrandt and Müller 1995). They were then stored in liquid N_2 over night. The next day, heads were fixed onto a metal block using Tissue Teck. The head capsule was opened and one antennal lobe of each bee was dissected under constant liquid N_2 cooling and transferred into a 100-μl glass capillary containing 10 μl extraction buffer (50 mmol l^{-1} TRIS-HCl, pH 7.5, 2 mmol l^{-1} EGTA and 10 mmol l^{-1} mercaptoethanol), cooled by liquid N_2. The antennal lobes were homogenised with a metal pistil on the surface of the frozen extraction buffer and stored in liquid N_2 till the start of the phosphorylation.

PKA activity was determined with phosphatase inhibitor (I-1), which was purified from bovine brain and used as the specific substrate (Hildebrandt and Müller 1995). Samples in the capillaries were thawed and immediately added to 10 μl phosphorylation mixture. This mixture contained 1 μCi (γ-^{32}P) ATP (5,000 Ci mmol^{-1}, 10 μM ATP, 20 mmol l^{-1} magnesium chloride ($MgCl_2$), 2 mmol l^{-1} EGTA and 10 mmol l^{-1} mercaptoethanol in 50 mmol l^{-1} TRIS-HCl, pH 7.5, and an aliquot of the heat-stable *Apis* PKA-specific substrate protein I-1, which was boiled for 2 min before use. After incubation for 30 s at room temperature, reactions were stopped by adding 5 μl SDS-PAGE loading buffer (500 mmol l^{-1} TRIS-HCl, pH 6.8, 5% mercaptoethanol, 5% SDS, 20% glycerol and 0.1% bromophenol blue). After SDS-PAGE and autoradiography, the autoradiographs were scanned using the program NIH Image. The relative PKA activity is calculated by dividing the density of the I-1 band (^{32}P incorporation into the PKA-specific substrate I-1) by the density of the corresponding lane (total ^{32}P incorporation). Values of each gel were normalised to allow comparisons.

Injection of 8-Br-cAMP

In this experiment, returning non-pollen foragers were captured at the hive entrance, cooled, mounted and fed as described above. One hour after feeding, the gustatory responsiveness of each bee was determined using water and the following sucrose concentrations: 0.1%, 0.3%, 1%, 3%, 10% and 30%. The total number of proboscis extensions of a bee constituted its individual GRS. As seven gustatory stimuli were used in this experiment, the GRS could vary between 0 and 7.

Injection of 8-Br-cAMP transiently increases PKA activity in the antennal lobes of honey bees, as has been shown by Hildebrandt and Müller (1995). Each bee was injected into the thorax with 1 μl of a defined concentration of 8-Br-cAMP, dissolved in Mobbs ringer solution (270 mmol l^{-1} NaCl, 3.2 mmol l^{-1} KCl, 1.2 mmol l^{-1} $CaCl_2$, 10 mmol l^{-1} MgCl, 10 mmol l^{-1} morpholinopropansulfonic acid, pH 7.4). Control bees were injected with 1 μl of Mobbs ringer solution. We tested the following concentrations of 8-Br-cAMP: 0.1 mmol l^{-1}, 0.3 mmol l^{-1} and 1.0 mmol l^{-1}. Gustatory responsiveness was measured again in each individual 15 min, 60 min, 120 min and 240 min after injection.

Injection of KT 5720

This experiment was done in exactly the same way as the injection of the PKA activator 8-Br-cAMP. Because the solvent of the PKA inhibitor KT 5720 was DMSO, we added 2% DMSO to the ringer solution in the controls. Earlier experiments by Müller (2000) have shown that injection of the PKA inhibitor KT 5720 decreases PKA activity significantly.

Feeding of 8-Br-cAMP to 1-day-old bees

Ben-Shahar et al. (2002) demonstrated that 4-day feeding of 8-Br-cAMP to 1-day-old bees significantly increases their PKA activity. To test the effect of 8-Br-cAMP on gustatory responsiveness we closely followed their feeding protocol. In our experiments, 1-day-old bees were removed from a comb maintained in a humidified incubator at 33°C. Bees were placed in cages of 16 cm×9 cm×6 cm and had free access to either 50% sucrose (control group) or to 1 mmol l^{-1} 8-Br-cAMP in 50% sucrose. The solutions were prepared freshly every day. The cages were stored in a humidified incubator maintained at 33°C. After 4 days, bees were individually removed from the cage and mounted in tubes as described above. Gustatory responsiveness was tested as described for the injection experiments. Testing started 1 h after mounting.

Feeding of 8-Br-cAMP to foragers

Returning non-pollen foragers were caught at the hive entrance and directly placed in the cages described above. Because in a number of pilot experiments the high concentration of 1 mmol l^{-1} 8-Br-cAMP was lethal for 100% of the foragers in the cage we reduced the 8-Br-cAMP concentration to 500 μmol l^{-1} in this experiment (see Results). This dose resulted in a survival rate which did not differ from that of sucrose-fed controls. After 4 days of feeding the bees with the PKA activator or the 50% sucrose solution, the foragers were individually mounted and tested for their gustatory responsiveness as described for the 1-day-old bees.

Statistics

For graphic display, the mean relative PKA activity and the standard errors of the means are shown for the different groups (Fig. 1). The mean value of subgroup 4 with very low gustatory responsiveness tested 30 min after feeding was defined as relative PKA activity of 1 and used to normalise the PKA activities of the other groups. The values of the PKA activity in each group were tested for normal distribution with two-tailed Kolmogorov-Smirnov test. Because the PKA activity of the different groups did not differ from normal distributions (group 1: $Z = 1.057$, group 2: $Z = 0.539$, group 4: $Z = 0.669$, group 5: $Z = 0.472$, $P > 0.05$, two-tailed Kolmogorov-Smirnov test), relative PKA activity was compared between bees with high or low GRS and between bees tested at different times using two-tailed *t*-tests.

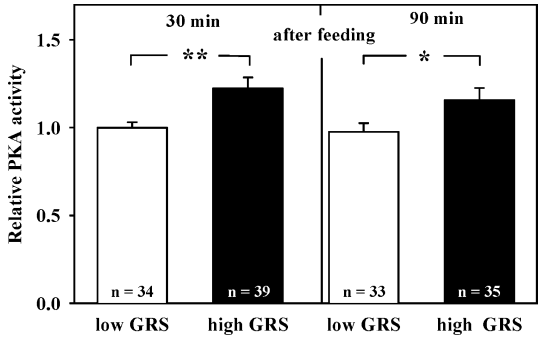


Fig. 1 Relative PKA activity in foragers with different gustatory responsiveness. Bees with low gustatory response scores (GRSs) of ≤ 1 only responded to sucrose concentrations equal to or greater than 30%. Bees with high gustatory response scores (GRS = 5) responded with a PER (proboscis extension response) to antennal stimulation with water and all sucrose concentrations. PKA activity was measured 30 min or 90 min after feeding. Mean GRS and standard errors of the means (SEM) are shown. The number (*n*) of bees tested is indicated for each group. Significant differences between the groups are marked by *asterisks* (*$P \le 0.05$, **$P \le 0.01$, two-tailed *t*-test)

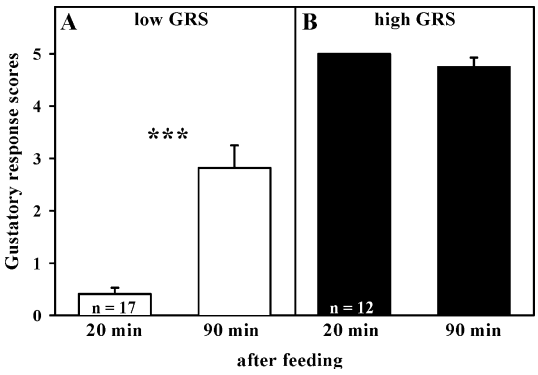


Fig. 2A,B Gustatory responsiveness 20 min and 90 min after feeding in bees with initially low GRS (≤ 1) and in bees with initially high scores (GRS = 5). **A** Individuals with low GRS 20 min after feeding become significantly more responsive 90 min after feeding (***$P \leq 0.001$, two-tailed Wilcoxon-test). **B** Bees with high GRS 20 min after feeding do not change their responsiveness 90 min after feeding. Means and SEM are shown. The number (n) of bees tested is indicated for each group

To illustrate the time dependence of gustatory responsiveness after feeding mean gustatory response scores and standard errors of the means are shown for groups 3 and 6 at 20 min and 90 min after feeding (Fig. 2). Within each group, GRS 90 min after feeding were compared with those measured 20 min after feeding using two tailed-Wilcoxon-tests, because the GRS 20 min after feeding differed significantly from normal distribution in both groups (group 3: no variance, group 6: $Z = 1.566$, $P \leq 0.05$) and the GRS 90 min after feeding differed in one group from a normal distribution (group 3: $Z = 1.696$, $P \leq 0.01$, group 6: $Z = 0.758$, $P > 0.05$, two-tailed Kolmogorov-Smirnov test).

For graphic display of changes in gustatory responsiveness after injection with the PKA activator 8-Br-cAMP or with the PKA inhibitor KT 5720 mean GRSs and standard errors of the means are shown (Figures 3 and 4). The effect of treatment with PKA activator or PKA inhibitor, the effect of time after injection and possible interaction effects of treatment and time were analysed by performing two-tailed Kruskall-Wallis H-tests (SPSS 11.0) on the aligned GRSs (for details of this method see Bortz et al. 2000), because most of the GRSs did not follow normal distribution. The evolution of GRSs with time after injection was analysed using two-tailed Friedman tests (SPSS 11.0).

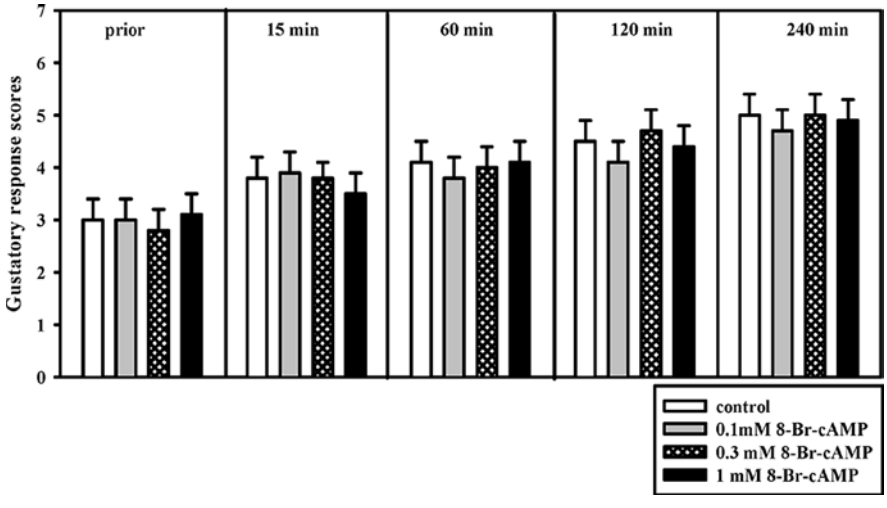
Gustatory responsiveness of 5-day-old bees and that of foragers fed with PKA activator in sucrose or sucrose alone was displayed as mean gustatory response scores with standard errors of the means (Fig. 5). The GRSs of sucrose-fed controls were compared with those of the treated groups using two-tailed Mann-Whitney U-tests, because the GRS of the different groups did not always follow a normal distribution (5-day-old bees sucrose: $Z = 2.166$, $P \leq 0.001$, 1 mmol l^{-1} 8-Br-cAMP: $Z = 1.731$, $P \leq 0.01$; foragers sucrose: $Z = 2.148$, $P \leq 0.001$, 500 μmol l^{-1} 8-Br-cAMP: $Z = 1.354$, $P > 0.05$, two-tailed Kolmogorov-Smirnov test).

Fig. 3 GRSs of bees injected with different concentrations of the PKA activator adenosine 3'5'-cyclic monophosphate 8-bromo-sodium salt (8-Br-cAMP) and control bees prior to injection and at different times after injection. The ordinates show mean GRS and SEM. GRSs were tested prior to injection and at the following times after injection: 15 min, 60 min, 120 min and 240 min. Bees were injected with one of the three different 8-Br-cAMP concentrations or ringer solution (*control*). The number of bees tested in each group is: control, $n = 42$; 0.1 mmol l^{-1} 8-Br-cAMP, $n = 41$; 0.3 mmol l^{-1} 8-Br-cAMP, $n = 41$; 1 mmol l^{-1} 8-Br-cAMP, $n = 40$. There were no significant differences between the GRS of the control bees and those of the bees treated with 8-Br-cAMP (for details of the statistics see text)

Results

Relative PKA activity in the antennal lobes of foragers with high or low gustatory responsiveness

Relative PKA activity differed significantly between bees with very low gustatory responsiveness and those with very high gustatory responsiveness (Fig. 1). Bees with high gustatory responsiveness displayed a significantly higher PKA activity than bees with very low gustatory responsiveness, regardless of the time when PKA activity was measured (at 30 min after feeding: $t = 3.17$, $df = 56$, $p \leq 0.01$, at 90 min after feeding: $t = 2.07$, $df = 66$, $p \leq 0.05$, two-tailed t-test). The time dependence of gustatory responsiveness of two parallel groups of bees (subgroups 3 and 6 in Table 1) was measured at 20 min and at 90 min after feeding to compare behavioural responses with PKA activity. Bees which had low gustatory responsiveness 20 min after feeding strongly increased their responsiveness until 90 min after



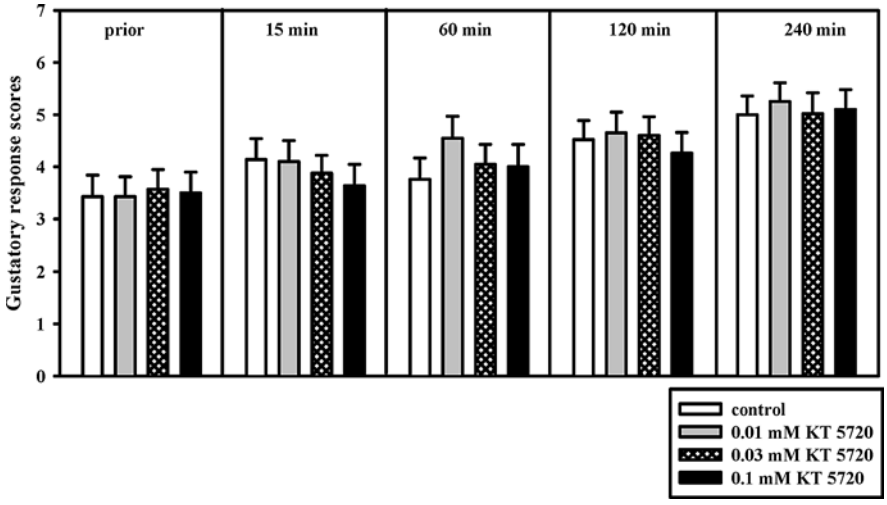


Fig. 4 GRSs of bees injected with different concentrations of the PKA inhibitor KT 5720 and control bees prior to injection and at different times after injection. The ordinates show mean GRS and SEM. GRSs were tested prior to injection and at the following times after injection: 15 min, 60 min, 120 min and 240 min. Bees were injected with one of the three different KT 5720 concentrations or ringer solution containing DMSO (*control*). The number of bees tested in each group is: control, $n = 42$; 0.01 mmol l^{-1} KT 5720, $n = 40$; 0.03 mmol l^{-1} KT 5720, $n = 42$; 0.1 mmol l^{-1} KT 5720, $n = 42$. There were no significant differences between the GRSs of the control bees and those of the bees treated with KT 5720 (for details of the statistics see text)

feeding (Fig. 2A, $Z = 3.34$, $P \leq 0.001$, two-tailed Wilcoxon-test), whereas bees which had a high responsiveness 20 min after feeding did not change their responsiveness significantly 90 min after feeding (Fig. 2B, $Z = 1.34$, $P > 0.05$, two-tailed Wilcoxon-test).

These results demonstrate a basal difference between the PKA activity of foragers with very high gustatory responsiveness and those with very low gustatory responsiveness. This PKA activity does not change in bees whose gustatory responsiveness increases significantly at 90 min after feeding.

The effects of a PKA activator on gustatory responsiveness in foragers

Injection of different concentrations of the PKA activator 8-Br-cAMP into the thorax of honey-bee foragers did not lead to significant effects on their gustatory responsiveness within the first 4 hours after treatment (Fig. 3). Gustatory response scores of some groups did not follow normal distributions. We therefore used two-tailed Kruskall-Wallis *H*-tests to analyse the effects of 8-Br-cAMP at different times after injection. These tests were performed separately for the effect of treatment, the effect of time and interaction effects on the aligned data (Bortz et al. 2000). Treatment with different concentrations of 8-Br-cAMP had no effect on gustatory response scores ($\chi^2 = 0.946$, $df = 3$, $P > 0.05$). In addition, there was no interaction effect of treatment and time on

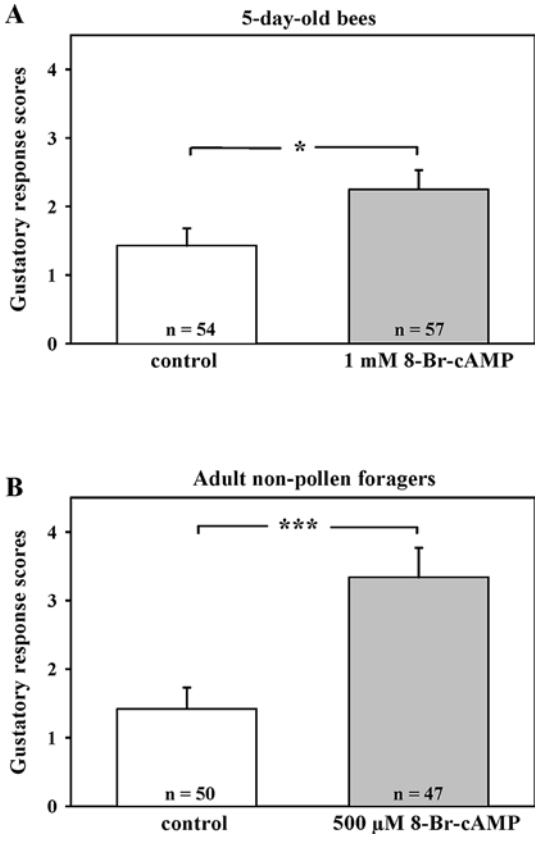


Fig. 5A,B Mean GRS and SEM for 5-day-old bees and adult non-pollen foragers that were fed with 8-Br-cAMP for 4 days. The number (*n*) of bees tested is indicated. **A** Five-day-old bees which had been fed with 50% sucrose (*control*) or 1 mmol l^{-1} 8-Br-cAMP in 50% sucrose for 4 days. Bees treated with 8-Br-cAMP had significantly higher GRSs than sucrose-fed control bees (*$P \leq 0.05$, two-tailed Mann-Whitney *U*-test). **B** Adult non-pollen foragers which had been fed with 50% sucrose (*control*) or 500 μmol l^{-1} 8-Br-cAMP in 50% sucrose for 4 days. Bees treated with 8-Br-cAMP had significantly higher GRSs than sucrose-fed control bees (***$P \leq 0.001$, two-tailed Mann-Whitney *U*-test)

GRS ($\chi^2 = 12.279$, $df = 15$, $P > 0.05$). Gustatory response scores were strongly affected by time ($\chi^2 = 78.687$, $df = 3$, $P \leq 0.001$). In all groups, gustatory responsiveness

increased with increasing time after injection (control: $\chi^2 = 21.50$, $n = 42$, $P \leq 0.001$; 0.1 mmol l^{-1} 8-Br-cAMP: $\chi^2 = 9.07$, $n = 41$, $P \leq 0.05$; 0.3 mmol l^{-1} 8-Br-cAMP: $\chi^2 = 17.11$, $n = 41$, $P \leq 0.001$; 1 mmol l^{-1} 8-Br-cAMP: $\chi^2 = 21.81$, $n = 40$, $P \leq 0.001$, two-tailed Friedman test).

The effects of PKA inhibitor on gustatory responsiveness in foragers

Injection of different concentrations of the PKA inhibitor KT 5720 did not affect gustatory responsiveness in the range of 4 h after application (Fig. 4). The GRS of some treatment groups did not follow normal distribution. Therefore, the effects of the KT 5720 treatment, time effects and interaction effects were tested using Kruskall Wallis H-tests on the aligned data (Bortz et al. 2000). The PKA inhibitor KT 5720, which was applied in three different concentrations, did not alter gustatory responsiveness ($\chi^2 = 0.684$, $df = 3$, $P > 0.05$). There was also no interaction effect of KT 5720 and time ($\chi^2 = 22.091$, $df = 15$, $P > 0.05$). But time had a significant effect on gustatory responsiveness ($\chi^2 = 84.669$, $df = 3$, $P \leq 0.001$). With increasing time after injection of the drugs or Ringer/DMSO, gustatory responsiveness increased significantly in all groups (control: $\chi^2 = 17.64$, $n = 42$, $P \leq 0.001$; 0.01 mmol l^{-1} KT 5720: $\chi^2 = 36.31$, $n = 40$, $P \leq 0.001$; 0.03 mmol l^{-1} KT 5720: $\chi^2 = 20.67$, $n = 42$, $P \leq 0.001$; 0.1 mmol l^{-1} KT 5720: $\chi^2 = 19.86$, $n = 42$, $P \leq 0.001$, two-tailed Friedman test).

Feeding PKA activator to 1-day-old bees

The injection of the PKA activator 8-Br-cAMP did not affect gustatory responsiveness in the first 4 h after treatment. In this experiment we therefore tested whether feeding of the same PKA activator for several days would lead to a change in gustatory responsiveness. The experiments of Ben-Shahar et al. (2002) have convincingly shown that 4-day feeding of the PKA activator 8-Br-cAMP to 1-day-old bees leads to a significant increase in the PKA activity of the then 5-day-old bees. Using the protocol of Ben-Shahar et al. (2002) we tested whether 4-day feeding of 1 mM 8-Br-cAMP would lead to a change in gustatory responsiveness.

Gustatory response scores of bees fed with sucrose or sucrose containing 8-Br-cAMP did not follow normal distribution (sucrose: $Z = 2.166$, $P \leq 0.001$, sucrose/1 mmol l^{-1} 8-Br-cAMP: $Z = 1.731$, $P \leq 0.01$). Therefore, non-parametric statistics were conducted. Feeding of 1 mM 8-Br-cAMP in a 50% sucrose solution for 4 days to 1-day-old bees led to a significant increase in gustatory responsiveness compared to control bees treated with sucrose (Fig. 5A, $Z = 2.50$, $n_{8\text{-Br-cAMP}} = 57$, $n_{\text{controls}} = 54$, $P \leq 0.05$; two-tailed Mann-Whitney *U*-test). Other behavioural effects of the 8-Br-cAMP treatment were not observable. The death rate of bees fed with sucrose (21.4%) did not differ significantly from that of bees treated with 1 mmol l^{-1} 8-Br-cAMP (18.6%).

Feeding PKA activator to adult foragers

Our experiments have shown that 4-day-feeding of 1 mmol l^{-1} 8-Br-cAMP increased gustatory responsiveness in newly emerged bees. The following experiment was to test whether the gustatory responsiveness of adult foragers can be modulated by the same protocol. However, the death rate of foragers fed with 1 mmol l^{-1} 8-Br-cAMP was 100% after 4 days of feeding, so that no effects on gustatory responsiveness could be measured. We therefore reduced the concentration to 500 μmol l^{-1} 8-Br-cAMP. This time, the death rate in bees fed with the PKA activator was with 7.5% not significantly different from that of sucrose-fed control bees with 5.7%.

Gustatory response scores of both groups did not follow normal distribution (sucrose: $Z = 2.148$, $P \leq 0.001$, sucrose/500 μmol l^{-1} 8-Br-cAMP: $Z = 1.354$, $P > 0.05$, two-tailed Kolmogorov-Smirnov test). Foragers fed with 500 μmol l^{-1} 8-Br-cAMP for 4 days displayed a significantly higher gustatory responsiveness than bees fed with sucrose alone (Fig. 5B, $Z = 3.31$, $n_{8\text{-Br-cAMP}} = 47$, $n_{\text{controls}} = 50$, $P \leq 0.001$; two-tailed Mann-Whitney *U*-test). This demonstrates that in adult foragers as in young hive bees, gustatory responsiveness can be modulated by several-day treatment with 8-Br-cAMP. The effects of long-term feeding with the PKA inhibitor KT 5720 on bee behaviour could not be tested, because the solvent DMSO is toxic when applied to bees for several days.

Discussion

Our experiments demonstrate that gustatory responsiveness of honey-bee foragers correlates with PKA activity in the antennal lobes. Individuals with high gustatory responsiveness show a higher PKA activity than bees with low responsiveness. This PKA activity is not affected by the increase in gustatory responsiveness which is apparent 90 min after feeding in bees with initially low responsiveness. Injection of the PKA activator 8-Br-cAMP or of the PKA inhibitor KT 5720 does not affect gustatory responsiveness up to 4 h after treatment. Only continuous feeding of the PKA activator for 4 days increases gustatory responsiveness in newly emerged bees and adult foragers.

These results enable us to distinguish between two different forms of gustatory responsiveness: *basal* and *transient* gustatory responsiveness. Basal gustatory responsiveness correlates with PKA activity in the antennal lobes (Fig. 1) and can be increased by feeding the PKA activator 8-Br-cAMP for several days (Fig. 5). The brain structures which are decisive for the determination of this basal gustatory responsiveness seem to

be the antennal lobes, because PKA activity measured in the central brain (brain minus optic lobes, antennal lobes and suboesophageal ganglia) does not correlate with gustatory responsiveness (M. Humphries, U. Müller, R.E. Page, personal communication). Some factors determining this basal gustatory responsiveness have already been identified. One factor which determines whether bees have a high or low gustatory responsiveness is genotype (Pankiw and Page 1999; Scheiner et al. 2001a, 2001b). Individuals of the "high-pollen-hoarding strain" of Page and Fondrk (1995) preferentially collect pollen and generally display a higher gustatory responsiveness than bees of the "low-pollen-hoarding strain", which mainly collect nectar. Foraging role in wild-type bees and in the two different genetic lines also affects gustatory responsiveness. Pollen foragers generally display a higher gustatory responsiveness than nectar foragers (Page et al. 1998; Scheiner et al. 1999, 2001b, 2003; Pankiw and Page 2000). The age of an individual also determines its gustatory responsiveness. Gustatory responsiveness generally increases with age (Pankiw and Page 1999).

The physiological mechanisms which regulate basal gustatory responsiveness are unknown, but it is conceivable that a long-term increase in PKA activity, such as that induced by 4-day feeding 8-Br-cAMP, may lead to a change in gene expression which could result in a different gustatory responsiveness. Indirect evidence for our hypothesis of changes in gene expression comes from the experiments of Ben-Shahar et al. (2002) who have shown that the transition from hive work to forager state in the honey bee is associated with an increase in the expression of the *for* gene, which encodes for protein kinase G (PKG). The level of PKG in foragers was significantly higher than that in young hive bees. The authors further demonstrated that 4-day feeding of 8-Br-cGMP, an activator of PKG, caused precocious foraging behaviour, presumably by a change in gene expression. The biogenic amine octopamine could be involved in the signal chain controlling basal gustatory responsiveness. Feeding octopamine to honey bees for several days, which elevates the brain levels of octopamine in the antennal lobes, increases their responsiveness to brood pheromone (Barron et al. 2002). It needs to be tested whether feeding octopamine for several days also increases gustatory responsiveness in honey bees.

Differences in basal gustatory responsiveness can have far-reaching effects. They can lead to differences in non-associative habituation and sensitisation of proboscis extension (Scheiner 2001) and in associative tactile and olfactory learning (Scheiner et al. 1999, 2001a, 2001b, 2003) by affecting the motivational state of a bee.

Transient gustatory responsiveness does not correlate with PKA activity and cannot be modulated with 8-Br-cAMP or KT 5720 within minutes or hours. The increase in gustatory responsiveness 90 min after feeding in initially unresponsive bees (Fig. 2) is not accompanied by a change in PKA activity. Injection of 8-Br-cAMP or KT 5720 does not affect transient gustatory responsiveness up to 4 h after treatment.

Feeding and foraging experience are factors which strongly influence transient gustatory responsiveness. Bees which have not been fed for several hours show an increased gustatory responsiveness (control group in Figs. 3 and 4; Page et al. 1998; Pankiw et al. 2001). Individual foragers show a higher gustatory responsiveness when they have collected 10% sucrose from a feeder than when they have been foraging on a 50% sucrose feeder (Pankiw et al. 2001).

The physiological mechanisms regulating transient gustatory responsiveness most likely involve biogenic amines but probably no PKA. It has been shown that gustatory responsiveness can be increased in the range of minutes by feeding or injecting octopamine or tyramine, while the injection of dopamine decreases gustatory responsiveness (Scheiner et al. 2002). When the brains of bees are depleted of biogenic amines by treatment with reserpine, responsiveness to sucrose is strongly reduced (Braun and Bicker 1992). Injection of the PKA activator 8-Br-cAMP has no effect on gustatory responsiveness in the first 4 h following treatment (Fig. 3).

Antennal stimulation with sucrose leads to a rapid increase in PKA activity which reaches its peak 0.5 s after stimulation and declines within 3 s (Hildebrandt and Müller 1995). The effect of the sucrose stimulus can be mimicked by local injection of octopamine or 8-Br-cAMP into the antennal lobe, which induces a strong increase in PKA activity 5 s after injection. The PKA activity returns to the level of control animals within 2 min (Hildebrandt and Müller 1995). Similarly, a short sucrose stimulation at the antenna can sensitise a bee for about 2 min (Menzel et al. 1991) but does not affect gustatory responsiveness in the range of minutes or hours (Page et al. 1998).

The effect of transient gustatory responsiveness on different forms of behaviour has hardly been studied. An increase in transient gustatory responsiveness as a result of low feeding status was shown to increase learning performance in associative olfactory learning (Menzel et al. 1991). It will be interesting to see whether an increase in transient gustatory responsiveness which is induced by biogenic amines can lead to a better learning performance and slower habituation, as has been shown for basal gustatory responsiveness (Scheiner et al. 1999, 2001a, 2001b, 2003; Scheiner 2001). It has been demonstrated that octopamine facilitates the storage and retrieval of learned information (Menzel et al. 1990) and rescues conditioning in reserpinised bees. But it needs to be analysed whether the facilitatory effect of octopamine in associative learning correlates with an increase in gustatory responsiveness or is independent of gustatory responsiveness.

Taken together, our findings comprise a new aspect of the general function of cAMP/PKA-mediated processes in behavioural plasticity. All functions of cAMP/PKA-regulated processes in neuronal and behavioural plasticity demonstrated so far require a characteristic

transient activation of the cAMP/PKA-pathway in the range of minutes to hours (Abel et al. 1997; Milner et al. 1998; Müller 2000). In contrast to this general characteristic of cAMP/PKA action, our findings show that gustatory responsiveness is only affected by long-lasting (days) activation of PKA. This suggests that modulation via the cAMP/PKA-cascade can occur in very different time windows. While transient PKA activation in the short-term range acts directly via covalent modification of target proteins, a long-lasting activation of the cAMP/PKA pathway most likely affects gene expression. Future experiments will have to identify the proteins which are affected by long-term activation of the cAMP/PKA-cascade and which are most likely involved in gustatory responsiveness.

Acknowledgements We would like to thank Dr. Gene Robinson and Dr. Yehuda Ben-Shahar for their suggestions regarding the feeding protocol of 8-Br-cAMP. We would also like to thank Dr. Robert Page who organised a workshop at the Santa Fe Institute, where the ideas for this study were discussed and the co-operation between different laboratory groups was initiated. This study was supported by a grant from the Deutsche Forschungsgemeinschaft (SFB515). The experiments we conducted comply with the current guidelines of Germany for the proper treatment of animals.

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