0014-4754/96/080762-07$1.50 + 0.20

Phosphorylation of cockroach antennal polypeptides: effects of second messengers and pheromonal blend

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Received 23 October 1995; accepted 1 February 1996

Abstract. In insect antennal extracts, Schleicher et al.[1] showed that protein kinase C (PKC) inhibitors abolish the transience of pheromone-induced rapid inositol trisphosphate responses, which suggests that pheromonal signals act on phosphorylation of specific proteins. To confirm this hypothesis, we studied the effects of second messengers and a pheromonal blend on phosphorylation of antennal proteins in the cockroach *Periplaneta americana*. Proteins from adult male antennae were phosphorylated in vitro in the presence of [γ^{32}P]adenosine triphosphate, then separated by SDS-polyacrylamide gel electrophoresis. Numerous phosphopolypeptides were visualized. The presence of Ca^{++}/calmodulin in the incubation medium resulted in increased phosphorylation of polypeptides with molecular weights of 38, 48, 51, 54 and 58 kDa. Stimulation of PKC by addition of Ca^{++} phosphatidylserine (PS)/phorbol myristate acetate (PMA) resulted in the appearance of three phosphopolypeptides of 36, 70 and 120 kDa. In the presence of cyclic adenosine monophosphate, two new major polypeptides of 46 and 42 kDa appeared; the latter polypeptide also appeared in the presence of cyclic guanosine monophosphate. Comparison with polypeptide composition of tissue from the cerci, leg, brain and fat body showed that the 36 and 48 kDa polypeptides were specific to antennae, whereas the 120 kDa polypeptide was also present in the adult brain. When antennae are subjected to pheromonal stimulation for 16 seconds prior to homogenization, in vitro phosphorylation of the 120, 70, 64 and 38 kDa polypeptides was inhibited, whereas phosphorylation of the 58, 54, 51 and 48 kDa polypeptides was strongly stimulated. It is noteworthy that a 107 kDa polypeptide was observed only after pheromonal stimulation by Ca^{++}/PS/PMA. Our findings suggest that Ca^{++}- and PKC-dependent protein phosphorylation systems play an important role in the transduction of pheromonal signals in antennae of male cockroach *P. americana*. We speculate that specific phosphoproteins may modulate sensitivity and signal amplification during the olfactory transduction process.
Key words. Protein phosphorylation; protein kinases; pheromones; second messengers; antennae; insect; cockroach.

The survival of any living organism depends largely on its ability to monitor the environment. This is particularly true for insects, since many aspects of both sexual and nonsexual behaviour depend on detection, discrimination and analysis of volatile chemicals by antennae.

In recent years, considerable progress has been made in understanding transduction of olfactory signals. Cyclic adenosine monophosphate (cAMP) and inositol 1,4,5-trisphosphate (IP_3) pathways have been implicated in the mechanism of action of odorants in vertebrates[2,3]. Involvement of these two pathways has also been demonstrated in odorant perception in one invertebrate, the lobster[4,5].

In insects, a complex cascade of molecular events takes place between the initial interaction of female sex pheromone components with male olfactory antennal receptors and the initiation of sexual behaviour. Recent studies have identified several pieces of this complicated puzzle:

1. In insect antennae, the specific activity of phospholipase C, the key enzyme of the phosphatidyl inositol pathway, is high[6,7].
2. In antennal extracts a blend of sex pheromones induces a rapid, transient rise, (within the subsecond time range[8]) in IP_3 mediated by G-protein[9,10].
3. A delayed, sustained increase in cyclic guanosine monophosphate (cGMP)[11] follows this 'IP_3 pulse'[8] and can activate the so-called AC1 channel described by Zufall and Hatt[12] and Zufall et al.[13].

The generally held hypothesis is that stimulation of the olfactory receptor leads to the increase in cAMP, cGMP, IP_3 or Ca^{++}. Any of these products could directly modulate the activity of an ion channel[14]. However, a more likely role for cGMP would be to mediate desensitization and/or other secondary modulatory reactions[15]. Since cAMP, cGMP and Ca^{++} are also components of the signal transduction pathways which result in activation of protein kinases and/or phosphatases, we hypothesize that phosphorylation of numerous proteins in the antennae is also affected as a consequence of odorant recognition. In this regard it is

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important to recall that protein phosphorylation/dephosphorylation is now a well-recognized mechanism of regulation of neuronal function[16].

The purpose of this study was to investigate our hypothesis. Using in vitro phosphorylation with [γ^{32}P]-adenosine triphosphate (ATP) and separation by SDS-polyacrylamide gel electrophoresis (PAGE), we investigated modulation of protein phosphorylation in response to second messengers and pheromones in cockroach antennae. Three sets of phosphorylation experiments were performed. In the first set, the effects of cyclic nucleotides, Ca^{++}/calmodulin and Ca^{++}/phosphatidylserine (PS)/phorbol myristate acetate (PMA), on phosphorylation of endogenous protein substrates were studied. In the second, tissue and sexual specificity of the phosphopolypeptides were determined. In the third, the effects of sexual pheromones on this phosphorylation/dephosphorylation system were documented.

Materials and methods

Chemicals

cAMP and cGMP were purchased from Boehringer-Mannheim. PS and PMA were obtained from Sigma. [γ^{32}P]ATP (specific activity 3000–4000 Ci/mmol) was from ICN.

Insects

Colonies of the cockroach *Periplaneta americana* were reared at 25–27 °C and 70% RH with access to dry dog food, apples and water. The photocycle was 12D:12L, the dark phase starting at 7.00 p.m. All experiments were performed during the first 8 h of the dark phase, the time of proximal sexual activity and sensitivity[17]. Newly emerged males were kept isolated from females in plastic boxes (three insects/box) pending phosphorylation experiments. To ascertain the role of sexual dimorphism and developmental stage in protein phosphorylation, last instar larvae and 20-day-old adult male and female *P. americana* were used throughout this study. Females were used mainly as a source for the natural pheromonal blend.

Pheromonal treatment

Preparation of pheromonal blend source. Virgin females (5 to 20 days old) were isolated from males before the imaginal moult, to prevent copulation, and were kept in groups of 5 to 10 animals. Sheets of filter paper were provided as shelters for at least two weeks. The pheromone-impregnated filter paper was then used as a source of the natural pheromonal blend according to the technique of Saas[18].

To ensure that the pheromone-impregnated paper had the same effect as a female, we carried out comparative behavioural tests with a reference test in which a receptive virgin female was introduced into a plastic box containing three quiescent 20-day-old males. In this reference test, males stopped maintenance behaviour and moved to the side of the box where the female was within 15 s. Then they moved around the area and fluttered their wings whenever they made contact with another animal. We verified that each sheet of pheromone-impregnated filter paper provoked the same response less than 15 s after introduction into a cage containing males.

Tissue and extract preparations

Insects were anaesthetized by CO_2. Antennae were removed, immediately frozen in nitrogen and stored at −20 °C pending use in protein phosphorylation experiments. To test specificity of polypeptides to different tissues, the cerci, fat body, brain and legs were dissected from the same male insects as antennae. To test pheromone effects, both intact antennae were removed from a single animal. Each antenna was placed in a closed petri dish. Testing consisted of exposure of one antenna to a sheet of pheromone-impregnated filter paper introduced into the dish, and the other antenna to a plain sheet of filter paper. After 15 s of exposure, antennae were immediately frozen by pouring liquid nitrogen into the dish.

Immediately before the phosphorylation experiments, frozen tissues were homogenized using a Potter homogenizer at −80 °C and homogenization buffer (20 mM Tris-HCl, 2 mM EDTA and 5 mM EGTA, adjusted to pH 7.5) was added. Homogenates were clarified by centrifugation at 200*g* for 5 min, and the supernatant was used immediately. All procedures were carried out at 4 °C. The protein content of the samples was estimated by the method of Bradford[19] using bovine serum albumin as a standard. Extracts were diluted to one milligram protein per milliliter.

'In vitro' phosphorylation and SDS-PAGE separation

In vitro phosphorylation was performed by incubation in a medium (final volume 50 µl) containing 50 mM phosphate buffer pH 7.0, 10 mM $MgCl_2$ and 10 µM [γ^{32}P]ATP (1.3 µCi). The reaction was initiated by adding 20 µl of biological extracts per lane. Incubation lasted 3 min at 37 °C. When used, exogenous modulators of the various protein kinases were added singly at the following final concentrations: 5 µM cAMP, 5 µM cGMP, 5 mM $CaCl_2$, 10 µg/ml calmodulin, 100 µg/ml PS and 1 µg/ml PMA. As described by Laemmli[20], the reaction was first stopped by adding 12.5 µl of a five-fold concentrated SDS sample buffer and heating for 3 min at 90 °C, and then incubation mixtures were submitted to SDS-PAGE in 12.5% gels using an SE 400 Sturdier 16-cm vertical slab gel unit (Hoefer Scientific Instruments, San Francisco, CA). Molecular weights were estimated using the following Bio-Rad protein

markers: myosin (200 kDa), β-galactosidase (116 kDa), phosphorylase (97 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21 kDa) and lysozyme (14 kDa). Gels were stained for 30 min in 0.2% Coomassie blue. After drying, labelled polypeptides were visualized by autoradiography at -20 °C for 48 h after exposure on Fuji RX films. All experiments were performed in triplicate.

Results

In vitro effects of putative modulators of protein phosphorylation. Over 12 phosphopolypeptides were routinely observed when no effector was added to the incubation medium (fig. 1). When an effector was added, a variety of patterns was observed depending on the modulator used (fig. 1 and table 1).

Addition of cAMP resulted in the appearance of two new phosphopolypeptides: one of 46 kDa (sometimes difficult to separate from the 45 kDa phosphopolypep-

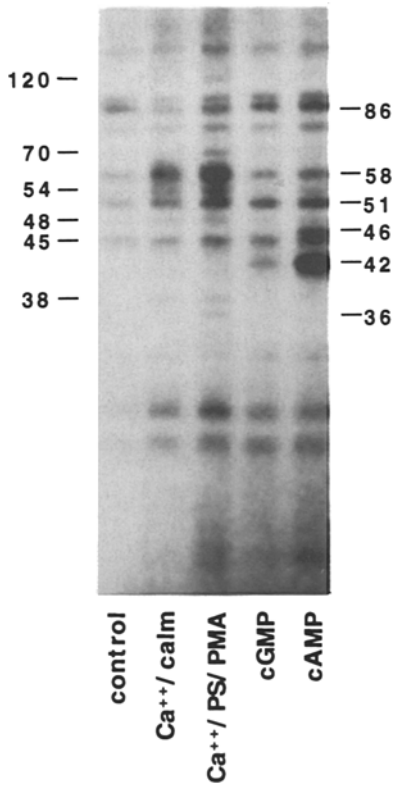

Figure 1. Effect of different effectors, Ca^{++}/calmodulin and Ca^{++}/PS/PMA, cGMP, cAMP, on the phosphorylation of endogenous antennae phosphopolypeptides. Samples from 1000*g* supernatants were submitted to in vitro phosphorylation in the presence of [γ^{32}P]ATP with or without (control) one of the following effectors: Ca^{++}/calmodulin, Ca^{++}/PS/PMA, cGMP, cAMP, as described in 'Materials and methods'. Polypeptides were separated by SDS-PAGE in a 12.5% polyacrylamide gel. Visualization of the phosphoproteins was achieved by autoradiography. Numbers on the right and left indicate molecular masses in kilodaltons.

Table 1. Effects of exogenous effectors of protein phosphorylation on endogenous substrates from *P. americana*.

Phosphopolypeptides (mol wt in kDa)	cAMP without pheromone	Ca^{++}/calm. without pheromone	Ca^{++}/PS/PMA without pheromone	Most active effector with pheromone	Tissue specificity with pheromone*
36			**appears**		**antennae**
38		appears	++	−	antennae, cerci, brain
42	appears +++			+	antennae, brain
45					antennae, cerci, brain
46	appears			+	antennae, brain
48	+/−	+	+	+++	**antennae**
51	+	+/−	+	+++	antennae, leg, cerci, brain
54		appears	++	+++	antennae, brain
58		+	++	+++	antennae, cerci, brain
64			+	−−	antennae, leg, cerci, brain
70			appears +	−−	antennae, cerci, brain
74			+/−	+/−	antennae, cerci, brain
86	+			+	antennae, cerci, brain
107				**appears** ++++	**antennae**
120			appears ++	−−−−	antennae, brain

+ to ++++ = weak to strong enhancement of phosphorylation.
− to −−−− = weak to strong inhibition.
*Using most active effector.

tide) and the other of 42 kDa. The latter phosphopolypeptide was also slightly phosphorylated in the presence of cGMP. Cyclic nucleotides also increased the phosphorylation of polypeptides at 86 and 51 kDa.

In the presence of Ca^{++}/calmodulin, two phosphopolypeptides of 38 and 54 kDa appeared, while phosphorylation of the 48 kDa and especially the 58 kDa polypeptide was stimulated.

When Ca^{++}/PS/PMA was used, we obtained the same pattern as with Ca^{++}/calmodulin, plus increased phosphorylation of the 51 kDa phosphopolypeptide, further stimulation of the 58 kDa phosphopolypeptide and the appearance of three new phosphopolypeptides at 36, 70 and 120 kDa.

Tissue specificity. The antenna is a heterogeneous organ containing polypeptides from the nervous system, cuticle and lymph. We attempted to classify antennal phosphopolypeptides by comparison with tissues coming from other organs from the same animal. Accordingly, phosphopolypeptides from the leg, fat body, cerci and brain were separated after in vitro phosphorylation (fig. 2 and table 1). Table 1 shows the results of the classification based on this data.

In control experiments, the 48 kDa phosphopolypeptide was detected only in antennae. All other phosphopolypeptides were present in one or more other tissues. The 91 kDa phosphopolypeptide was present in all five tested tissues. Although not always strongly phosphorylated, the 64 and 51 kDa polypeptides were observed in several tissues: antennae, leg, cerci and brain for the former, and antennae, brain and leg for the latter. The 86, 74, 58 and 45 kDa polypeptides were present in antennae, cerci and brain. Since the same amount (20 µg) of protein was used in these experiments, it appears that protein kinase activity and/or protein substrates were very high in antennae as compared with the other organs. This difference was especially great in comparison to the leg and fat body.

The 42 and 46 kDa phosphopolypeptides which appeared in antennae in the presence of cAMP were also detected in the brain under the same conditions. In the presence of Ca^{++}/PS/PMA, all polypeptides whose phosphorylation was stimulated in the antennae were also found in the brain and/or cerci except the 36 kDa protein, which was highly specific to antennae.

Sexual dimorphism. Our data demonstrate that there is a sex-related dimorphism in the phosphopolypeptides expressed by adult cockroaches, but not by larvae (fig. 3). Autoradiograms of larvae of both sexes and of adult males seem to be similar, and differ from those of adult females with respect to protein phosphorylation. Phosphorylation of a 100 kDa polypeptide is specific to

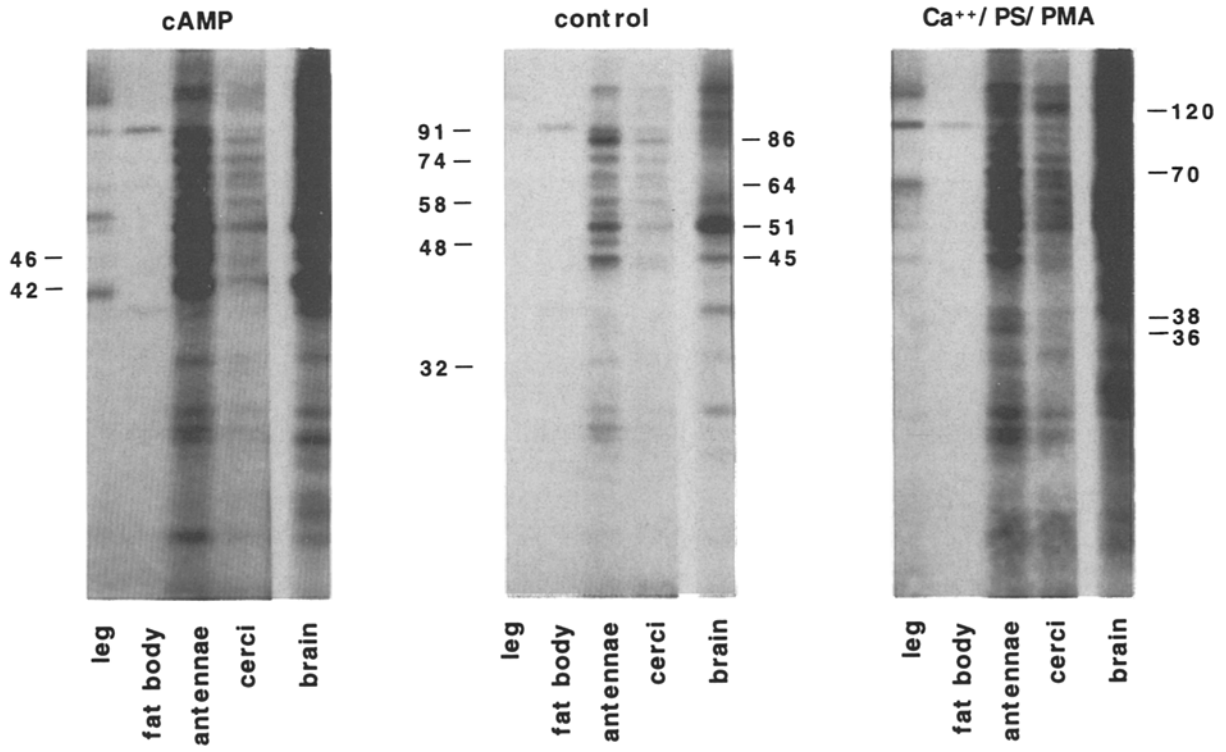

Figure 2. Phosphorylation of endogenous polypeptides in different *P. americana* tissues. In vitro protein phosphorylation was performed in the presence of [γ^{32}P]ATP, in 1000*g* supernatant samples containing 20 µg of protein extract from leg, fat body, antennae, cerci and brain prepared as described in 'Materials and methods'. Phosphorylation took place without (control, middle) or with either cAMP (left) or Ca^{++}/PS/PMA (right). Due to the strong labelling in the brain, the duration of autoradiography was 24 h rather than 48 h in the other tissues. Numbers on the right and left indicate molecular masses in kilodaltons.

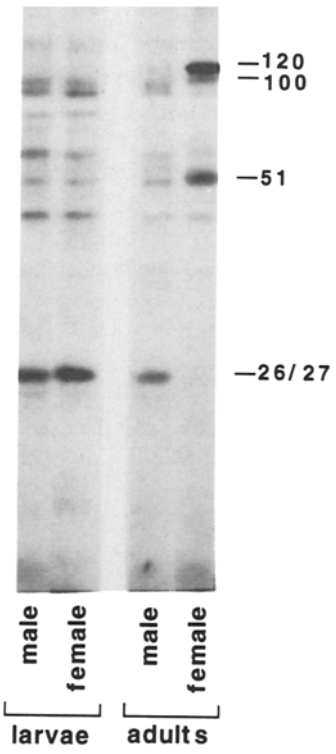

Figure 3. Comparison of polypeptides phosphorylated in antennae according to the sex and developmental stage. Samples from 1000*g* antennal supernatants from larvae or adults and from males or females were phosphorylated under standard conditions, and proteins were separated by SDS-PAGE. Autoradiograms were performed as indicated in 'Materials and methods'. Molecular weights (in kilodaltons) of the phosphopolypeptides exhibiting the greatest differences are indicated in the right margin of the figure.

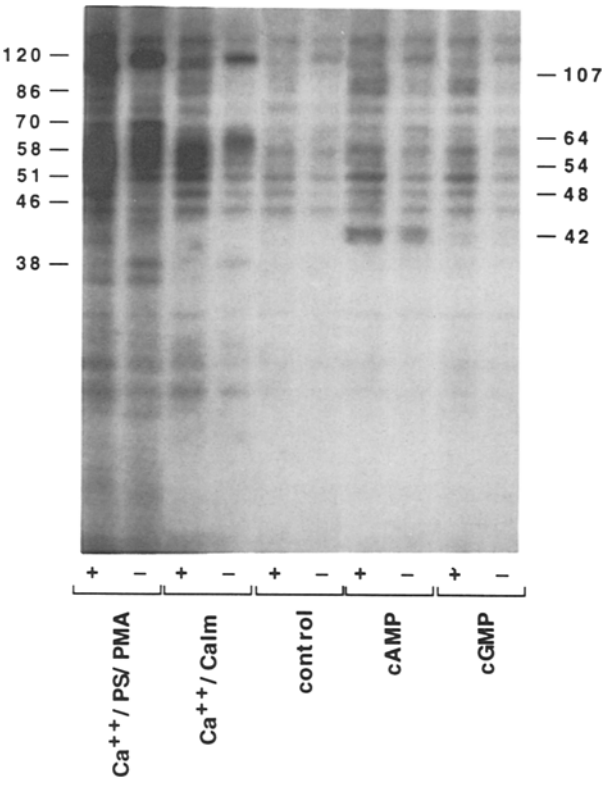

Figure 4. Pheromone-induced changes in male antennae phosphoprotein phosphorylation. Intact male antennae were exposed (+) or not (−) to a sheet of filter paper impregnated with female pheromones for 15 s. Then 1000*g* antennal supernatants were prepared and subjected to in vitro phosphorylation with or without (control) one of the following modulators: Ca^{++}/PS/PMA, Ca^{++}/calmodulin, cAMP or cGMP. Separation of labelled phosphopolypeptides and autoradiography was achieved by SDS-PAGE, as indicated in 'Materials and methods'. Numbers on the right and left indicate molecular masses in kilodaltons.

female adults, while phosphorylation of the 26–27 kDa doublet is specific to the male. The 120 and 51 kDa polypeptides were strongly labelled in female adults.

Pheromonal effects. Figure 4 and Table 1 show the results of experiments using isolated antennae exposed or not to filter papers impregnated with pheromones. In control experiments using no modulator, phosphorylation of the 58, 51 and 48 kDa polypeptides was increased by exposure to pheromones, whereas phosphorylation of the 120 kDa was decreased. Stimulation of the 58, 54, 51 and 48 kDa polypeptides was even greater using Ca^{++} and greater again using Ca^{++}/PS/PMA. With Ca^{++} and Ca^{++}/PS/PMA we also noted a decrease in phosphorylation of the 70, 64 and 38 kDa polypeptides, and above all the appearance of a 107 kDa phosphopolypeptide not previously detected. In the presence of cyclic nucleotides, the effects of the pheromonal blend were less spectacular, but phosphorylation of the 42, 46 and 86 kDa polypeptides was stimulated.

Discussion and conclusion

Major advances have been made in understanding the molecular mechanisms involved in olfactory transduction in neuronal tissues of vertebrates[21]. In invertebrates knowledge about the second messenger cascade is much more limited, but current evidence is sufficient to assume that transduction of olfactory signals involves a diversity of mechanisms, including the phospholipase C pathway[7,22,23] as well as the cAMP-[5,24] and cGMP-linked pathways[11,12]. Since second messengers are generally activators of protein kinases, and since PKC inhibitors abolish the transience of pheromone-induced rapid IP_3 responses[1], we hypothesized and then demonstrated in this study that reversible protein phosphorylation is also implicated.

It is difficult to compare our data with those in the literature. We are the first group to have separated and characterized the endogenous substrates of protein kinases in the insect antenna. This was done under conditions promoting phophorylation (phosphate buffer). We detected around 12 major phosphopolypeptides in the antenna of male *P. americana* and determined their molecular weights after separation by SDS-PAGE. Addition of second messengers such as cAMP, cGMP, Ca^{++}/calmodulin or Ca^{++}/PS/PMA to the incubation

medium to activate the different protein kinase families enabled us to ascertain the pathways involved in the regulation of phosphorylation of these polypeptides. Incubation with modulators also revealed phosphorylation of new polypeptides, e.g. the 46 and 42 kDa polypeptides which are substrates of protein kinase, the 54 and 38 kDa polypeptides which are substrates of kinase depending on Ca^{++}/calmodulin, and the 120, 70 and 36 kDa protein which are substrates of PKC.

Antennae are composed of various types of tissue (sensorial neurons, glial cells, epidermis and cuticle) and lymph, which although not a tissue also contains transport polypeptides, e.g. odorant binding polypeptides[25] or enzymatic polypeptides, e.g. esterases[26]. It is thus likely that the phosphopolypeptides detected in the antenna are also present in other organs and in particular in nervous tissue (brain and other sensory organs) and that few phosphopolypeptides are specific to the antenna. However our findings showed two phosphopolypeptides found only in antenna extracts, the 36 and 48 kDa polypeptides. This was confirmed by unsuccessful attempts to visualize these polypeptides even in other tissues in the presence of effectors. Phosphorylation of the 36 kDa polypeptide depends exclusively on Ca^{++}/phospholipid-activated PKC while that of the 48 kDa protein is also regulated by Ca^{++}/calmodulin and cAMP.

The 120, 54, 42 and 46 kDa polypeptides were phosphorylated only in the antenna and brain. Under our experimental conditions, phosphorylation of the 120 and 54 kDa polypeptides was again dependent on Ca^{++}, whereas phosphorylation of the 42 and 46 kDa polypeptides took place only in the presence of cAMP, which indicates that these polypeptides are substrates only of protein kinase. Judging from the width of the band, the 42 kDa protein could be a doublet, and we speculate that it may be the same as the 42 kDa protein reported in the brain of another Orthoptera, the cricket *Acheta domesticus*[27]. Interestingly, Hesse and Marmé[28] reported that cAMP stimulated a 44 kDa phosphoprotein in the head of *Drosophila melanogaster*. Kelly[29] also detected a 46 kDa protein in extracts from the head of *D. melanogaster*, the phosphorylation of which is regulated by cAMP, and speculated that it might be the regulatory subunit of cAMP-dependent protein kinase. The role of the 42 and 46 kDa phosphopolypeptides in the transduction of the pheromonal signal remains unclear, since the participation of the cAMP-dependent pathway in the transduction of odours has not been documented in insects[8].

The 91, 64 and 51 kDa polypeptides were found in phosphorylated form in four of the five tissues that we studied. The 51 kDa protein which was found in all samples tested is nonspecific to the order, since we observed a phosphopolypeptide of similar molecular weight in the brain of the cricket[27]. In both the cockroach and the cricket, this protein was a substrate of Ca^{++}/calmodulin-dependent kinases. However in our model the protein was phosphorylated by several protein kinases, including those dependent on cyclic nucleotides. The 64 kDa protein was phosphorylated in the antennae, cerci and legs but could not be detected in the brain. This suggests that it might be a soluble cuticle protein.

An especially important finding in this study was that after exposure of intact antennae to the pheromone-impregnated filter paper, labelling of the 120 kDa protein was almost completely inhibited, while that of the 107 kDa appeared. These effects illustrate the key role of PKC and its phosphorylated substrates in the transduction of the pheromonal message in insect antenna.

Under our experimental conditions the effect of the pheromonal blend seems to be complex. When assayed in the presence of Ca^{++}/PS/PMA, phosphorylation of the 70 kDa polypeptide is decreased, whereas phosphorylation of the 58, 54 and 48 kDa polypeptides is increased. Conversely, in the presence of cyclic nucleotides, phosphorylation is not significantly affected. This finding suggests that the Ca^{++} pathway plays a dominant role in response to pheromone.

In insects it is now clear that pheromone induces changes in production of second messengers (especially IP_3). In addition, our findings suggest that pheromonal response, signal amplification and desensitization involve specific phosphopolypeptides. Further study is needed to identify the proteins affected by pheromones and to understand fully how their phosphorylation/dephosphorylation is regulated.

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