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Are Polyamines Involved in Olfaction? An EAG and Biochemical Study in *Periplaneta americana* Antennae

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Abstract

Polyamines have been implicated in modulation of numerous cell functions. The purpose of this study was to assess the role of polyamines in intracellular regulation of insect antenna. Analysis of study data showed two main findings. First, *in vivo* treatment with the polyamine synthesis inhibitor α-difluoromethyl-ornithine enhanced the sensitivity of male *Periplaneta americana* antenna to female pheromonal blend. Secondly, polyamine modulated phosphorylation of several antennary proteins including two found exclusively in antenna (30 and 48 kDa). In both of these exclusive antennary proteins, phosphorylation changed after stimulation with the pheromonal blend. These results suggest that polyamines play a regulatory role in detection of female pheromonal blend and that modulation of protein phosphorylation is one of the mechanisms involved in this regulation.

Introduction

First described over 300 years ago (van Leeuwenhoek, 1678), polyamines form an ubiquitous group of low-molecular-weight aliphatic amines synthesized as intermediate products during catabolism of arginin (Seiler, 1994). The most common polyamines, i.e. spermidine, spermine, and their precursor putrescine, are present in significant quantities in all living cells [for a review, see (Morgan and Wallace, 1994)]. In mammals, polyamines are found in both neurons and glial cells. The neuron is the main cellular locus of ornithine decarboxylase (ODC), a key enzyme of the polyamine synthesis pathway (Bernstein and Müller, 1999).

Polyamines have generated special interest because of their wide-ranging ability to modulate biological activities underlying cellular signalling. In the last two decades, many prokaryotic and eukaryotic physiological and cellular functions including egg development in insects (Kogan and Hagedorn, 2000) have been shown to be polyamine-dependent [for a review, see (Cohen, 1998)]. Polyamine function has also been studied at the molecular level [for a review, see (Igarashi and Kashiwagi, 2000)]. Previous studies have shown that polyamines not only enhance transcription, processing and incorporation of RNA in ribosomes (Blair, 1985) but also bind to DNA and regulate gene expression (Feuerstein *et al*., 1991). Involvement of polyamines in regulation of post-translational processes has also been reported. Our previous results in the neural tissue of insects have shown that polyamines can modulate intracellular protein phosphorylation patterns both positively and negatively (Degrelle *et al*., 1994).

Polyamines may act via protein kinases and phosphoprotein phosphatases (Morgan, 1990). In this regard, *in vitro* experiments show that casein kinase II (CK II) (Filhol *et al*., 1991), a Ser/Thr protein kinase present in the nucleus and cytoplasm of all eukaryotic cells, is markedly activated by polyamines. Leroy *et al*. provided chemical evidence for the presence of a major spermine binding domain on the β subunit of CK II (Leroy *et al*., 1995). Polyamines have also been shown to stimulate phosphoprotein phosphatase 1 and 2A (Sjöholm and Honkanen, 2000). Spermine at physiological concentrations prevents inactivation of protein kinase C (PKC) by reducing its insertion into the hydrophobic core of the membrane (Monti *et al*., 1994).

Polyamines have also been implicated in modulation of *N*-methyl-D-aspartate (NMDA) receptors (Romano and Williams, 1994) involved in various forms of synaptic plasticity including some types of associative long-term potentiation and long-term depression which may underlie learning and memory (Collingridge and Lester, 1989).

Intracellular polyamines modulate many ion channels (Williams, 1997) including the strongly inwardly rectifying potassium channels (Lopatin *et al*., 1995; Lee *et al*., 1999;

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Guo and Lu, 2000a). Polyamines have also been shown to block cGMP-gated channels of the retinal (Guo and Lu, 2000b) and olfactory membrane (Lynch, 1999). Odour detection by olfactory receptor neurons in mammals is mediated by Golf protein-coupled receptors. When stimulated, these receptors induce a rapid increase in cAMP, which in turn activates the olfactory-specific cyclic nucleotide-gated (CNG) channel. Activation of these channels initiates neuronal depolarization and mediates calcium (Ca^{2+}) influx (Menini, 1999).

In insects, olfactory transduction probably involves an IP_3 cascade which leads to opening of IP_3-dependent Ca^{2+} channels (Stengl *et al*., 1999). PKC (Maida *et al*., 2000) and Ca^{2+}-dependent protein phosphorylation (Schleicher *et al*., 1994; Renucci *et al*., 1996) have been implicated in this process. However, to our knowledge, there is no direct evidence for either polyamine-dependent regulation of odour detection or the presence or action of polyamines in insect antennae. This study was designed to determine first whether polyamines play a direct or indirect role in the control of the olfactory transduction pathway in insects and second whether they modulate phosphorylation of antennary proteins. The first question was addressed by electroantennography (EAG) following treatment with α-difluoromethyl-ornithine (α-DFMO), a known specific inhibitor of the polyamine synthesis pathway. The second question was studied by sodium dodecyl sulphate (SDS)–polyacrylamide gel electrophoresis (PAGE) after *in vitro* phosphorylation of antenna extracts with or without addition of spermine.

Materials and methods

Chemicals

Spermine was purchased from Sigma Aldrich Chimie (l'Isle d'Abeau Chesnes, France) [γ-^{32}P]ATP (sp. act.: >4000 Ci/mmol) from ICN Pharmaceuticals (Orsay, France), CK II from Upstate Biotechnology Incorporated (Euromedex, Souffelweyersheim, France), and α-DFMO from Ilex, Inc. (San Antonio, TX).

Insects

Pending EAG recordings and phosphorylation experiments, newly emerged *Periplaneta americana* males were isolated from females in 11 × 4 cm (diameter × depth) plastic dishes (three insects per dish). Dishes were kept at 25–27C with a relative humidity of 70% and photocycle of 12 h dark:12 h light (dark phase beginning at 08:00 h). Insects were allowed free access to dry dog food and water. All experiments were performed during the first 8 h of the dark phase when sexual activity and sensitivity are optimal (Hawkins and Rust, 1977).

Preparation of pheromonal blend

The natural pheromonal blend was obtained according to the impregnation technique of Saas (Saas, 1983). Before the imaginal moult, 5–10 virgin females were isolated from males in boxes containing Whatman no. 42 filter paper as shelter material. After at least 2 weeks, the filter paper was removed and used as a source of natural pheromonal blend. Comparative behavioural tests were carried out to ascertain that the paper had the same attractive effect as a living female. In the reference test, a receptive virgin female was introduced into a plastic box containing three quiescent 20-day-old males. Within 15 s, males stopped maintenance behaviour and crossed over to the female side of the box. Wing fluttering was observed whenever a male made contact with another animal. The same behaviour was observed within 15 s after introduction of each sheet of filter paper impregnated with the pheromonal blend.

Electroantennography

Biological material

Fourteen α-DFMO-treated males and 14 untreated males were used. α-DFMO treatment was performed on 10-day-old animals by replacing drinking water with a 3% α-DFMO solution. Treatment lasted for 5 days and the solution was renewed every 2 days. Continuous video surveillance was used to ascertain that treated as well as control animals drank regularly.

Odour cartridges

Odour cartridges used for EAG were prepared by sealing pieces (0.8 × 8 cm) of filter paper impregnated with the pheromonal blend in glass tubes. Control cartridges were made with pieces of clean filter paper.

Experimental set-up

After cold anaesthesia, antennae were removed using microsurgical scissors and mounted between two stainless steel electrodes (Syntech, Hilversum, The Netherlands). Contact was maintained using electrically conductive gel (Spectra 360, Parker Laboratories, Orange NJ, USA) which also prolonged antenna viability by preventing loss of haemolymph. The pheromonal blend was applied to the antenna by blowing a pulse of air (920 ml/min) through the cartridge into a metal conduit carrying a continuous stream of humidified carbon-filtered air (20 ml/s) over the antenna. To prevent 'pressure shock', the air blown through the cartridge was diverted from the stream in the main conduit so that the total air flow over the antenna was constant. A recovery period of 90 s was allowed between each stimulus to avoid receptor adaptation. Odour cartridges were replaced after stimulation of two or three antennae. Antenna response was expressed in millivolts (mV). Amplified data was stored on a computer via an interface unit and data acquisition card (IDAC, Syntech).

Data analysis

Pre-processing of data was performed using on EAG 2.3b software (Syntech). For further analysis, data were

transferred from EAG 2.3b to SchoolStat v2.0.8. Responses were expressed as means in millivolts. Significant differences (P<0.05) between two population were evaluated using the nonparametric Mann–Whitney test.

Protein phosphorylation

Tissue and extract preparations

After cold anaesthesia, both antennae were removed from 15-day-old males. One antenna from each animal was exposed to the paper impregnated with the pheromonal blend and the other antenna was exposed to clean filter paper. After 15 s of exposure, antennae were frozen in nitrogen and stored at –20°C pending protein phosphorylation experiments. To allow identification of polypeptides specific to the antenna by comparison with those contained in other organs, the cerci, fat body, brain and legs were also dissected from the same insects and stored under the same conditions.

Immediately before phosphorylation experiments, frozen tissues were powdered at –80°C in a tissue grinder (Kontes/Fisher, Illkirch, France) and homogenized in a buffer containing 20 mM Tris-HCl, 2 mM ethylenediaminetetraacetic acid (EDTA) and 5 mM ethyleneglycol-bis(beta-aminoethyl-ether)-*N*,*N*,*N′*,*N′*-tetraacetic acid (EGTA), adjusted to pH 7.5. Homogenates were clarified by centrifugation at 200 *g* for 5 min and the supernatants used immediately. All procedures were carried out at 4C. Protein content in samples was estimated as described by Bradford (Bradford, 1976) using bovine serum albumin as the standard. Extracts were diluted to 1 µg protein per µl.

b. 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 µg of biological extract per lane. Incubation lasted 3 min at 37°C. When added, spermine was used at a final concentration of 2 mM and CK II at 10 mU. If necessary, inactivation of protein kinases was performed by heating samples to 55°C for 5 min. The reaction was stopped by addition of 12.5 µl of a five-fold concentrated SDS-sample buffer and heating to 90°C for 3 min as described by Laemmli (Laemmli, 1970). Incubation mixtures were then submitted to SDS–PAGE in 12.5% gels. After drying, labelled polypeptides were visualized by autoradiography using Fuji RX films. The duration of autoradiography was 48 h for all samples except brain extracts (<24 h). All experiments were performed in triplicate.

Results

Effect of α-DFMO on male *P. americana* antenna sensitivity to female pheromonal blend

As shown in Figure 1, the response (in mV) of antennae

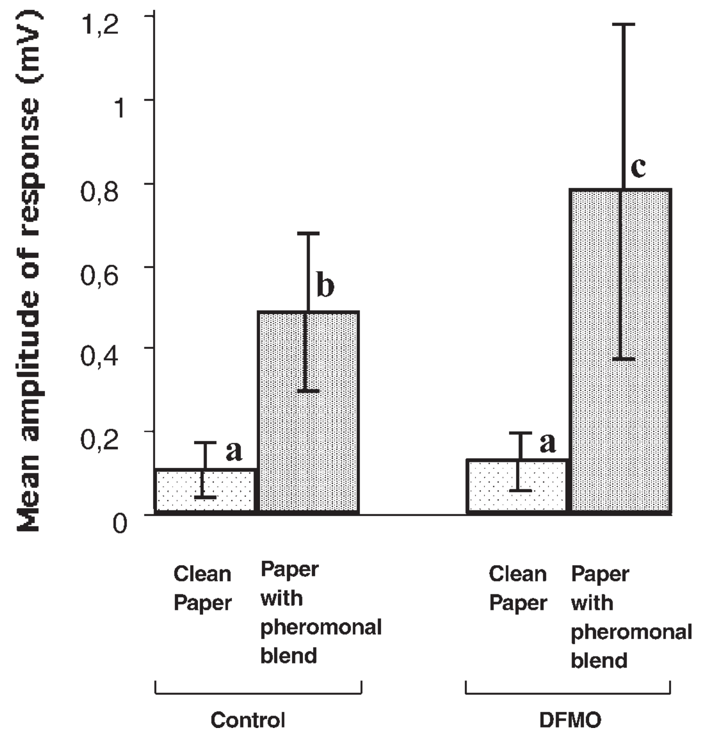

Figure 1 Comparison of mean EAG responses to the pheromonal blend. As described in Materials and methods, EAG recordings were performed after exposure to filter paper impregnated with the pheromonal blend or clean filter paper. We also compared antenna from male *P. americana* that received only water (control) or a 3% α-DFMO solution. *P < 0.05 (Mann–Whitney test). The value between the vertical bars corresponds to standard deviation. n = 14 for each population. Different letters indicate significantly different results.

from control males was significantly higher after exposure to cartridges containing filter paper impregnated with the female pheromonal blend (0.490 ± 0.19) than clean filter paper (0.110 ± 0.06). This was also the case for antennae from the α-DFMO-treated males (0.799 ± 0.4 versus 0.130 ± 0.07). Further comparison showed that the mean response to the female pheromonal blend was significantly higher for the 14 antennae from α-DFMO-treated males than for the 14 antennae from untreated control males (0.779 ± 0.4 versus 0.49 ± 0.19, Mann–Whitney U-test, P = 0.021). This finding demonstrates that α-DFMO treatment significantly enhanced the sensitivity of male antenna to the female pheromonal blend.

In vitro effects of spermine and CK II on male antennal protein phosphorylation

As shown in Figure 2, lane B, over 12 phosphopolypeptides were routinely observed in control antennae. Addition of 2 mM spermine (lane A) enhanced phosphorylation of the 22, 23, 26/27, 30, 33, 37, 48, 51, 54 and 120 kDa phosphopolypeptides. On the other hand, phosphorylation of the 45, 86 and 91 kDa phosphopolypeptides decreased. Heat inactivation of protein kinases (lane C) led to the

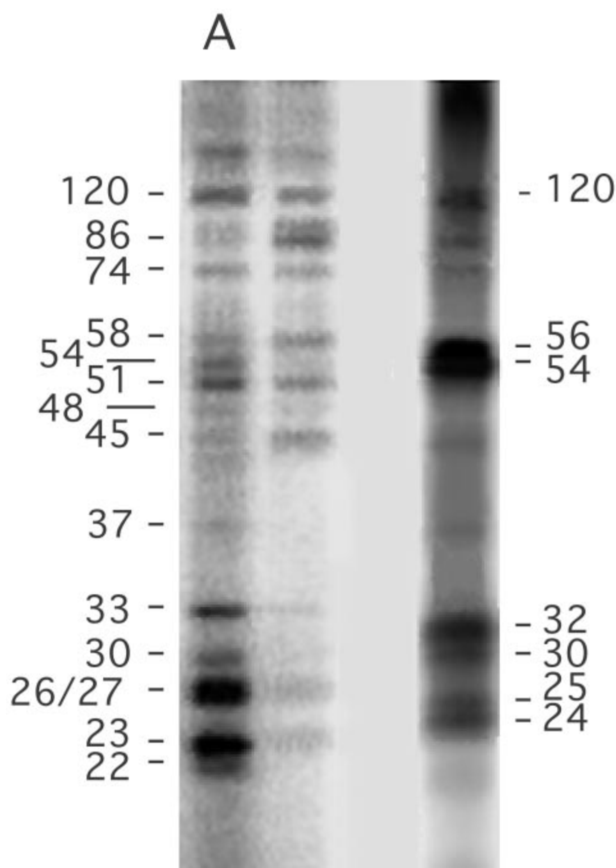

Figure 2 Effect of spermine and CK II on phosphorylation of endogenous male antennae phosphopeptides. *In vitro* phosphorylation in the presence of [γ-^{32}P]ATP was performed on the native extract in the presence (lane A) or absence (lane B) of 2 mM spermine and on the inactivated extract in the presence (lane D) or absence (lane C) of CK II. Numbers on the right and left indicate molecular mass in kilodaltons (also shown on Figures 3 and 4).

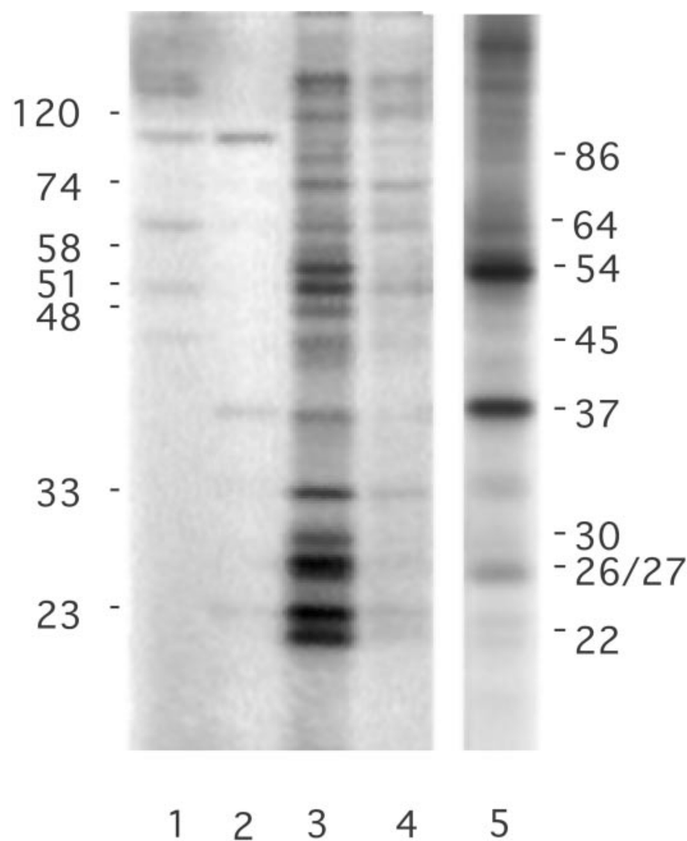

Figure 3 Phosphorylation of endogenous polypeptides in various *P. americana* tissues. *In vitro* protein phosphorylation was performed in the presence of [γ-^{32}P]ATP and spermine, in 1000 *g* supernatant samples containing 20 μg of protein extract from the leg (1), fat body (2), antennae (3), cerci (4) and brain (5) prepared as described in Materials and methods. Separation by SDS–PAGE and autoradiography were performed as described in Materials and methods. Due to strong labelling in brain extracts, the duration of autoradiography was shortened.

disappearance of the phosphorylated bands while addition of CK II after protein kinase inactivation (lane D) led to the appearance of the 24, 25, 30, 32, 54, 56, 74, 86 and 120 kDa phosphopolypeptides. So, these results shows that the 30, 54, 86 and 120 kDa proteins whose phosphorylation is stimulated by spermine are also substrates for CK II.

Tissue specificity

Since the antenna contains nervous tissue, cuticle, muscle and lymph, we attempted to identify phosphoproteins specific to the antenna by comparison with other types of tissue from the same animal. Accordingly, phosphoproteins from the leg, fat body, cerci and brain were separated after *in vitro* phosphorylation in the presence of spermine. As shown in Figure 3, the 30 and 48 kDa phosphoproteins were detected only in antennae. Although not always strongly phosphorylated, all other phosphoproteins were present in one or more other tissues. Since the same amount (20 μg) of protein was used in all experiments, it appears that protein kinase activity and/or protein substrates were high in antennae as compared with other organs. This difference was especially great in comparison with the leg, cerci and fat body but not with the brain, especially with regard to the 37 and 51 kDa phosphoproteins.

Effect of pheromonal blend

Figure 4 shows the results of experiments using isolated male antennae exposed to either filter paper impregnated with the female pheromonal blend or clean filter paper. In control experiments using no modulator, phosphorylation of the 58, 51, 48 and 45 kDa polypeptides increased after exposure to the pheromonal blend whereas phosphorylation of the 120 kDa polypeptide decreased. When phosphorylation was performed in the presence of spermine, pre-exposure to the pheromonal blend strongly enhanced phosphorylation of the 54, 48 and 33 kDa polypeptides. Conversely, phosphorylation of the specific antennal

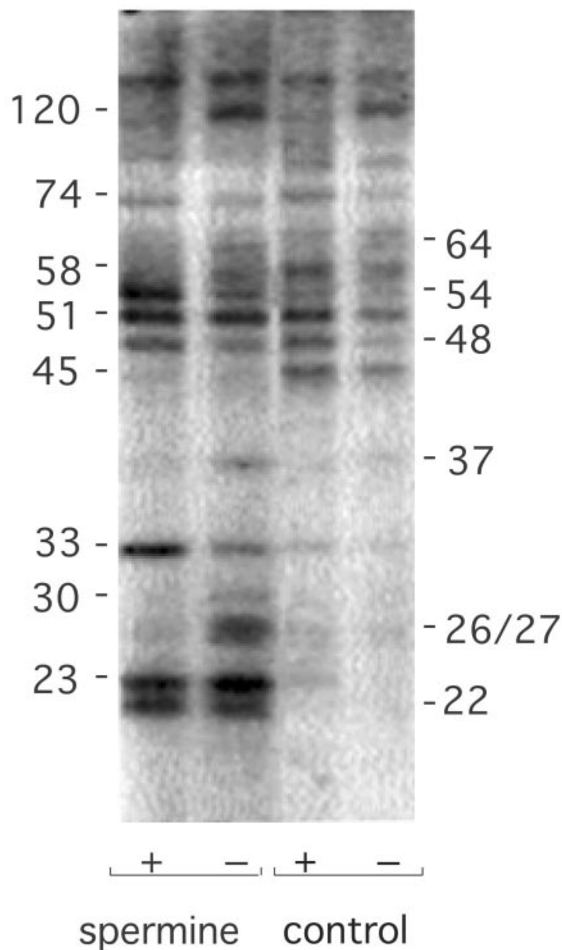

Figure 4 Pheromone-induced changes in male antenna phosphoprotein phosphorylation. Intact male antennae were exposed for 15 s to filter paper impregnated (+) or not (–) with female pheromonal blend. Following exposure, 1000 *g* antennal supernatants were prepared and subjected to *in vitro* phosphorylation in the presence or absence of spermine. Separation of labelled phosphoproteins by SDS–PAGE and autoradiography was achieved, as described in Materials and methods.

30 kDa polypeptide as well as of the 23, 27–28, 37 and 120 kDa polypeptides decreased.

Discussion

The EAG data described in this report demonstrate enhancement of the sensitivity of insect antenna to female pheromonal blend after treatment with α-DFMO. According to the literature [for a review, see (Cohen, 1998)], α-DFMO is a specific inhibitor of ODC, i.e. the rate-limiting enzyme for polyamine synthesis in mammals. In insects, our previous studies confirmed that administration of α-DFMO in drinking water for a 5 day period resulted in a decrease in polyamine levels in fat body and neural tissue (Cayre *et al.*, 1996). These findings strongly suggest that polyamines play a regulatory role in olfactory detection.

The mechanism by which a decrease in polyamine level leads to increase in olfactory sensitivity is still not clear. A possible explanation could involve action of polyamines on ion channels. Indeed, endogenous polyamines, in particular spermine, have been found to block or modulate several types of ion channels (Lopatin *et al.*, 1995; Lee *et al.*, 1999; Lu and Ding, 1999). In the olfactory system, Lynch showed that polyamines induced strong inward rectification in the CNG channel (Lynch, 1999). To our knowledge all data have been obtained on mammals and nothing is known of the action of polyamines on insect CNG channels.

Another mechanism that could be involved to some extent in the polyamine effect is protein phosphorylation. It has been demonstrated that CNG channels are regulated not only through the direct action of cyclic nucleotides, but also via phosphorylation catalysed by PKC (Müller *et al.*, 1998). Data presented in this study demonstrate that polyamines can modulate protein phosphorylation in antennae. However, the affected proteins have not been fully characterized and none of them exhibit a subunit with a molecular weight corresponding to any of the known CNG channels. Thus it can be assumed either that polyamines do not modulate the phosphorylation of these channels or that the channels affected by polyamines have not yet been characterized. The second possibility may open an avenue for further research, especially in insects.

Schleicher *et al.* showed that stimulation of *Heliotis virescens* antennal preparations using a pheromonal blend led to the stimulation of the phosphorylation of two proteins at 55 and 70 kDa (Schleicher *et al.*, 1994). This stimulation was suppressed by inhibitors of PKC. Although they were not characterized by those authors, these proteins might be the same as the 54 and 70 kDa shown to be substrates for PKC and to be affected by a pheromonal blend in antennal preparations of *P. americana* (Renucci *et al.*, 1996). The present work demonstrates that the 54 kDa protein is also a substrate for CK II. This finding leads us to speculate that the 54 protein may be the β subunit of the tubulin. In this regard it is noteworthy that the 53.5/54 kDa protein was clearly identified as the tubulin β subunit in previous experiments using a specific antibody in the brain of the cricket *Acheta domesticus* and bee *Apis mellifera* (Degrelle, 1996). Since the importance of multisite phosphorylation of key proteins is well documented (Cohen, 2000), our finding that spermine inhibits and stimulates phosphorylation of the 48 and 86 kDa proteins, respectively, is of interest because previous findings have shown that the 48 kDa protein is stimulated by cyclic nucleotides while the 86 kDa protein is stimulated by PKC activators (Renucci *et al.*, 1996).

Although direct involvement of polyamines in regulation of cell functions has been well documented (Cohen, 1998), the role of polyamines as intermediates in the transduction of hormonal and other regulatory signals remains highly conjectural. Cayre *et al.* implicated polyamines in transduction of the juvenile hormone message in insect neural tissue (Cayre *et al.*, 1997). Regulation of protein phos-

phorylation by polyamines has been observed in mammals (Cochet and Chambaz, 1983) and insects (Combest and Gilbert, 1992). The underlying mechanism involves stimulation of CK II in a process implicating binding of polyamine to a specific site on the regulatory subunit (Leroy *et al.*, 1997). We have demonstrated a regulation of CK II activity according to the hormonal status in the insect nervous system (Degrelle *et al.*, 1997).

Although we did not actually demonstrate an effect of polyamines on the phosphorylation of potential subunits of CNG channels, our data suggests a possible regulatory role via proteins with specialized functions. Phosphorylation of the 30 and 48 kDa proteins which are specific to antennae is affected by polyamines and changes after the detection of the pheromonal blend. Our results also suggest that modification of protein status via CK II is probably one of the mechanisms involved in this regulation. To definitively prove the presented hypothesis, the involved proteins must be identified, localized to the olfactory receptor neurons and compared to receptor potentials and nerve impulse responses recorded from single pheromone-sensitive neurons.

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