J Comp Physiol B (1990) 160:99–103

Journal of Comparative Physiology B Biochemical, Systemic, and Environmental Physiology

Pheromone-induced stimulation of inositol-trisphosphate formation in insect antennae is mediated by G-proteins

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Accepted October 27, 1989

Summary. Phospholipase C in the antennae of *Periplaneta americana* was stimulated by pheromones in a GTP-dependent manner. The enzyme activity was potentiated by hydrolysis-resistant analogs of GTP and decreased by GDP analogs. Guanine nucleotide binding regulatory proteins (G-proteins) in antennal preparations were identified by bacterial toxin-catalyzed ADP-ribosylation and immunoreactivity with antibodies of designed specificity. The stimulatory effect of pheromones on inositol phosphate formation was completely blocked by pertussis toxin suggesting that the pheromone action was mediated via specific G-proteins.

Key words: Phospholipase – G-protein – Periplaneta – Periplanone – Olfaction

Introduction

The molecular mechanisms underlying olfactory signal transduction are still poorly understood. In spite of considerable efforts and progress in vertebrate olfactory biochemistry (Lancet and Pace 1987) the sequence of molecular events between the initial interaction of odorants with olfactory receptors and generation of an action potential remains speculative, although there is circumstantial evidence suggesting the participation of adenylate cyclase and cAMP in olfactory signal transduction in vertebrates (Pace et al. 1985; Sklar et al. 1986).

In many ways, insects are ideal models for exploring the details of olfactory reception and transduction since most insects show a strong male-female dimorphism in the response to pheromones which appears to be highly active, specific and selective (Kaissling 1986). There is as yet no direct biochemical evidence regarding the molecular mechanisms involved in the transductory events of the chemoreceptive responses in the antennae of insects. In a recent study we have demonstrated that insect antennae contain a high specific activity of phospholipase C (Breer et al. 1989), the key enzyme of the phosphatidylinositol pathway (Berridge 1984; Rhee et al. 1989). This enzyme is stimulated by low doses of pheromones in a tissue- and species-specific manner, suggesting the involvement of specific receptor molecules for pheromones (Boekhoff et al. 1989). In several transduction systems coupling between the activated receptor and the effector enzyme is mediated by a G-protein that is active in the GTP-bound form and reverts to the inactive state upon hydrolysis of the bound GTP to GDP (Cassel et al. 1977; Cockcroft and Gomperts 1985). In the present study evidence is presented that guanine nucleotide regulatory proteins couple pheromone receptors in a stimulatory way to antennal phospholipase C.

Abbreviations: *G-protein* guanine nucleotide-binding regulatory protein; *IP_3* inositol 1,4,5-trisphosphate; *GTPS* guanine-5′-[thio]-triphosphate; *GDPβS* guanine-5′-[β-thio]-diphosphate; *NAD* nicotinic acid adenine dinucleotide; *SDS-PAGE* sodium dodecylsulfate polyacrylamide gel electrophoresis; *DTT* dithiothreitol; *MOPS* 3-(N-morpholino)propanesulfonic acid; *PIPES* piperazine-N,N′-bis[2-ethanesulfonic acid]; *DMSO* dimethyl-sulfoxide.

Materials and methods

Materials. Adult cockroaches (*Periplaneta americana*) were obtained from local suppliers [^{32}P]-NAD was obtained from New England Nuclear. [^{3}H]-labelled inositol phosphatidyl-bisphosphate (19.3 Ci·mmol^{-1}) and the IP_3 radioligand assay kit were purchased from Amersham. Cholera toxin was received from Sigma; pertussis toxin from List Biological Laboratories. Antisera against G-protein subunits were generously provided by Dr. S. Mumby. Periplanone B was a generous gift from Prof. J. Boeckh.

Phospholipase C assay. Cockroaches were anaesthetized by cooling at 4 °C; antennae were dissected and homogenized in assay buffer containing 50 m*M* MOPS, 200 m*M* NaCl, 10 m*M* EGTA, 2.5 m*M* $MgCl_2$, 1 m*M* DTT, pH 6.9. The homogenate was centrifuged at 800 × *g* for 20 min and the supernatant assayed for enzyme activity. Tritiated phosphatidyl-bisphosphate was dried under N_2 and dispersed by vortexing and sonication in assay buffer containing 0.5% (w/v) sodium cholate. The reaction was initiated by mixing the

enzyme sample with the sonicated substrate supplemented by 10 μM phosphatidylinositol, 1 mM ATP, 10 μM GTP, and $CaCl_2$ required to give a free Ca^{2+} concentration of 0.05 μM. The reaction was performed at 25 °C for 30 s and terminated by adding 1 ml chloroform/methanol/HCl (100:100:0.6), followed by 0.3 ml 1 N HCl containing 5 mM EGTA. The radiolabelled reaction products in the aqueous phase were determined by scintillation counting. Protein content of the samples was estimated by the procedure of Bradford (1986).

Determination of inositol-1,4,5-trisphosphate (IP_3). Samples of an homogenate from cockroach antennae (100–150 µg protein) were incubated at 25 °C in 50 mM MOPS, 200 mM NaCl, 10 mM EGTA, 0.05% (w/v) sodium cholate, 2.5 mM $MgCl_2$, 1 mM DTT, 1 mM ATP, and $CaCl_2$ to give a free Ca^{2+} concentration of 0.05 μM; periplanone B, dissolved in DMSO, and GTP were added as indicated. After incubation for 10 s, 500 µl ice-cold $HClO_4$ (10%, v/v) was added to the incubation mixture. After 10 min on ice, the samples were centrifuged for 5 min at 800 × g. 400 µl supernatant was transferred to a separate tube containing 100 µl 10 mM EDTA (pH 7.0). The samples were neutralized by adding 300 µl of a solution prepared from 1,1,2-trichlorotrifluoroethane (Freon) and tri-n-octylamine (1:1, v/v). After centrifugation for 1 min at 800 × g, 100 µl of the upper phase was removed. An equal volume of assay medium (0.1 M TRIS-HCl, pH 9.0, 4 mM EDTA, 4 mg·ml^{-1} bovine serum albumin), plus D-myo-[^{3}H]-inositol 1,4,5-triphosphate (approx. 3000 cpm), and appropriate amounts of binding proteins were added and the mixture incubated for 15 min on ice. The samples were centrifuged for 3 min at 1000 × g and the supernatant removed. Radioactivity in the pellet was determined by liquid scintillation counting; non-specifically bound radioactivity was estimated in the presence of 3 nmol unlabelled inositol-trisphosphate.

Before determining the effect of bacterial toxins, preactivated A promoters of cholera toxin and pertussis toxin were prepared by incubating the cholera toxin (300 µg·ml^{-1}) for 30 min at 37 °C in 15 mM TRIS-HCl (pH 7.5) containing 25 mM dithiothreitol and pertussis toxin (100 µg·ml^{-1}) with 1 mM ATP and 20 mM DTT for 30 min at 37 °C. Enzyme solutions (100 µl) were preincubated with 20 µl preactivated cholera toxin (30 µg·ml^{-1}) or preactivated pertussis toxin (1 µg·ml^{-1}) in the presence of 1 mM NAD for 15 min at 37 °C, followed by 15 min at 4 °C. IP_3 formation was immediately assayed. Controls were run without toxins.

ADP-ribosylation. Labelling of G-proteins by ADP-ribosylation was carried out according to the procedure of Ribeiro-Neto et al. (1985) using cholera and pertussis toxin to catalyze the reaction. Antennae were homogenized at 4 °C in buffer containing 50 mM PIPES, pH 6.5, 0.01% Triton X-100, 5 mM EDTA, 5 mM EGTA, 1 mM PMSF. The homogenate was centrifuged at 800 × g for 10 min at 4 °C; the resulting pellet was re-homogenized and centrifuged again. The combined supernatants were centrifuged at 17000 × g for 20 min at 4 °C. The resulting pellet was resuspended and incubated with preincubated cholera toxin (30 µg·ml^{-1}) in the reaction buffer containing 25 mM TRIS-HCl, pH 7.5, 5 mM ATP, 1 mM GTP, 10 mM thymidine, 1 mM EDTA, 15 μM [^{32}P]-NAD (about 10^6 cpm), or with pertussis toxin (1.5 µg·ml^{-1}) in 250 mM potassium phosphate-buffered saline (pH 7.5), 1 mM EDTA, 10 mM $MgCl_2$, 10 mM DTT, 10 mM ATP, 100 μM GTP, and 15 μM [^{32}P]-NAD (about 10^6 cpm). The reaction was terminated after 30 min by adding ice-cold trichloric acid (20%). After 20 min on ice, the mixture was centrifuged at 7000 × g for 20 min. The resulting pellets were treated with ethyl ether, dried and solubilized in electrophoresis sample buffer. Polypeptides were electrophoretically separated on SDS-polyacrylamide slab gels (12.5%). The gels were dried and exposed to Kodak-X-AR films for autoradiographic identification of labeled polypeptides.

Immunoblotting. Antennal membrane proteins were separated by electrophoresis on SDS-polyacrylamide (12.5%) slab gels and electrophoretically transferred onto nitrocellulose membrane. The nitrocellulose filters were dried and stained with Ponceau S in order to assess the quality of the transfer. Subsequently, the filters were probed for putative G-proteins using antisera raised against synthetic peptides of bovine G-proteins which are reactive with the α-subunits (A 569) or with the β-subunit (U 49) of G-proteins (Mumby et al. 1986). After blocking the non-specific binding sites by incubation with 1% bovine serum albumin, the blots were incubated for 1 h at room temperature with the primary antibodies at a dilution of 1:5000. Bound antibodies were visualized by an alkaline phosphatase labelled second antibody using 5-bromo-4-chloro-3-indolylphosphate (4 mg·ml^{-1}) and nitroblue tetrazolium (1 mg·ml^{-1}) as reaction medium.

Results

Phospholipase C catalyzes hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP_2) producing the potential second messengers diacylglycerol and inositol-1,4,5-trisphosphate (IP_3). The activity and specificity of phospholipase C in the antennae of *Periplaneta americana* was determined by incubating homogenates from cockroach antennae with exogenous radiolabelled PIP_2 tritiated at the 2-position of the inositol ring. The basal activity of phospholipase C in antennal preparations from *Periplaneta* (2.66 µmol·min^{-1}·mg protein^{-1}) was moderately stimulated by nanomolar concentrations of the cockroach specific sex pheromone periplanone B (the carrier solution, 0.01% DMSO, showed no effect), and also by micromolar concentrations of GTP (Table 1). In the presence of pheromone plus GTP the enzyme activity was synergistically increased by more than threefold. The potentiation of the pheromone stimulated hydrolysis of PIP_2 by GTP suggests that guanine nucleotide regulatory proteins may couple pheromone receptors to phospholipase C.

In order to elucidate if the antennal phospholipase C is indeed under the control of G-proteins, the effects of exogenous GTP as well as GTPS, and GDPβS, the hydrolysis-resistent analogs of GTP and GDP, respectively (Eckstein et al. 1979), were analysed. Since measurements of the IP_3 levels appear to be more sensitive indicators of the phospholipase C activity (Bradford and Rubin 1985) the concentrations of IP_3 were determined using a radioreceptor assay (Challis et al. 1988; Palmer

Table 1. Pheromone-induced stimulation of phospholipase C in antennal preparations from *Periplaneta americana*. The specific activity of phospholipase C in antennal preparations from *Periplaneta americana* was determined by incubation for 30 s at 25 °C with 0.05 μM free Ca^{2+} and 1 mM ATP. Data represent the means of 3 experiments ± S.D.

Additions	µmol inositol phosphate·min^{-1}·mg protein^{-1}
–	2.66 ± 0.54
DMSO (0.01%)	2.58 ± 0.42
Periplanone B [6 nM]	4.14 ± 0.35
GTP (1 μM)	3.81 ± 0.36
Periplanone B [6 nM] plus GTP [1 μM]	9.31 ± 1.08

Table 2. Effect of guanine nucleotides on inositol trisphosphate formation in antennal preparations from *Periplaneta americana*. Antennal preparations from cockroaches were incubated for 30 s in the presence of various guanine nucleotides. IP_3 concentration was determined using a radioreceptor assay. Data are the mean of n experiments ±S.D.

Assay conditions	pmol $IP_3 \cdot mg\ protein^{-1} \pm$S.D.	n
Control	106 ± 2.2	(4)
1 μM GTP	298 ± 38	(4)
1 μM GTPS	5300 ±1356	(3)
1 μM GDPβS	56.4± 5.6	(3)

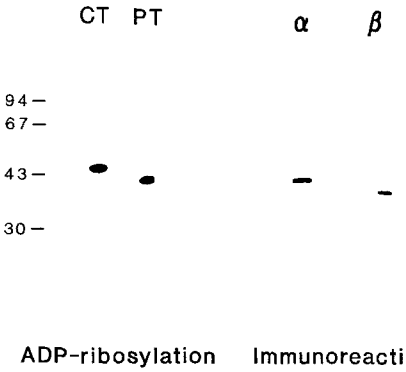

Fig. 1. Identification of putative G-proteins in antennal preparations from *Periplaneta americana* by ADP-ribosylation and immunoreactivity. Autoradiographic analysis of radiolabelled antennal polypeptides resulting from bacterial toxin catalyzed ADP-ribosylation. Antennal preparations were incubated with [^{32}P]-NAD in the presence of preactivated cholera toxin (CT) or pertussis toxin (PT) as described in the text. After electrophoretic separation under denaturing conditions on SDS-PAGE, labelled polypeptides were visualized by autoradiography. Western blot analysis of immunological crossreactivity of antennal polypeptides with antibodies reactive with α-subunits (α) or β-subunits (β) of G-proteins (Mumby et al. 1986). Polypeptides were separated by SDS-PAGE and transfered onto nitrocellulose. Strips of nitrocellulose were incubated with the antisera of designed specificity at a dilution of 1:5000 for 1 h at room temperature. The reactive polypeptide bands were visualized by an alkaline phosphatase-labelled second antibody

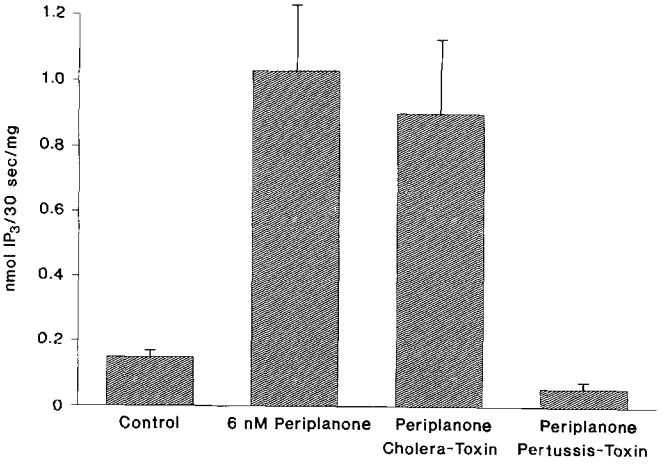

Fig. 2. Effects of bacterial toxins pretreatment on pheromone-stimulated generation of inositol-trisphosphate in antennal preparations from *Periplaneta*. Antennal preparations were preincubated with preactivated cholera toxin or pertussis toxin as described in the text. Subsequently, the pheromone-induced formation of IP_3 was assayed by incubating with 6 n*M* periplanone B for 30 s. Data are the means of 3–5 experiments ±SD

et al. 1989). As can be seen in Table 2, incubation with 1 μ*M* GTP for 30 s moderately increased the concentration of IP_3. By application of hydrolysis-resistent GTP, the signal-terminating step is prevented, leading to a persistently activated effector system. Addition of GTPS (1 μ*M*), consequently caused a considerably elevated IP_3 level. In contrast, GDPβS, which competitively inhibits the binding of GTP to G-proteins (Eckstein et al. 1979), markedly attenuated the IP_3 concentration. These results are considered as an indication that phospholipase C in the antennae of *Periplaneta* is under the stringent control of a G-protein that is inactive when it contains bound GDPβS, and is persistently active when the bound nucleotide is the hydrolysis-resistent analog GTPS.

An important question concerning receptor-mediated regulation of inositol phosphate formation centres on the identity of the putative regulatory protein that subserves the trans-membrane signalling function in this system. Two different approaches were applied to identify any G-proteins in the antennae of *Periplaneta americana*, the bacterial toxin catalyzed ADP-ribosylation and the immunoreactivity with antisera of designed specificity. In Fig. 1 it can be seen that in the presence of cholera toxin a polypeptide band (M_r 45000) was ADP-ribosylated, probably the α-subunit of a G_s-protein; whereas a different polypeptide (M_r 40000) was labelled by pertussis toxin catalyzed ADP-ribosylation, which may either represent the α-subunit of a G_i- or G_0-protein. The nature of the putative constituents of antennal G-proteins was further analyzed by immunoblotting using subunit-specific antibodies. In membrane preparations from *Periplaneta americana* only the 40 kDa band showed crossreactivity with the α-subunit specific antibodies under standard conditions (Fig. 1); only under more intensive labelling conditions could the 45 kDa band also be visualized. Immunoblotting with an antiserum reactive with the β-subunit of G-proteins revealed an immunoreactive band at 36 kDa. Thus, appropriate G-proteins are present in the antennal preparations from *Periplaneta americana* as has recently been demonstrated for other insect species (Breer et al. 1988).

Due to their ADP-ribosyltransferase activity cholera toxin, as well as pertussis toxin, change the action of the modified G-proteins. Whereas ADP-ribosylation of G_s by cholera toxin results in activation of the protein and an increased sensitivity to GTP, ADP-ribosylation of G-proteins by pertussis toxin causes an inactivation apparently because of decreased nucleotide binding (Gilman 1987). Thus, the bacterial toxins can be applied to evaluate which type of guanine nucleotide regulatory protein mediates the pheromone-induced phospholipase C stimulation. As demonstrated in Fig. 2, incubation

of antennal preparation with cholera toxin did not alter the sensitivity of IP_3 formation in response to pheromone stimulation (6 n*M* periplanone B), whereas pertussis toxin almost completely blocked the pheromone induced accumulation of IP_3, indicating that a pertussis toxin-sensitive G-protein apparently couples pheromone receptors to the phosphatidylinositol pathway in antennal cells.

Discussion

One of the major criteria for determining whether an effector system is regulated by a G-protein is the demonstration of a response to GTP or its non-hydrolysable analogs (Gilman 1987). The results described here provide firm evidence that the phospholipase C in antennal preparations from *Periplaneta americana* can be activated by GTP analogues and is inhibitable by GDPβS; furthermore, the stimulation of this enzyme by specific pheromones is essentially GTP-dependent. Thus, it is apparent that the pheromone effect on phospholipase C is mediated via activated guanine nucleotide binding proteins. As for the transducin in the visual system (Stryer 1986), the G-protein of the pheromone-perceptive cells in insect antennae may play a pivotal role in signal amplification that could be the basis of the extreme sensitivity of pheromone detection in insects (Boeckh et al. 1965; Kaissling 1986).

Pertussis toxin-induced ADP-ribosylation prevents the coupling of the G-protein with the activated receptor and also stabilizes this G-protein in its associated, GDP-bound state, thus preventing its action on effector systems (Ui et al. 1984). The effect of bacterial toxin on a particular effector system is therefore considered as another indication of regulation by a GTP-binding protein (Bourne and Stryer 1986). The finding that pertussis toxin completely suppressed the formation of IP_3 induced by periplanone B indicates that the pheromone effect is mediated by a pertussis toxin-sensitive G-protein and adds the antennal cells of *Periplaneta* to the list of cells where pertussis toxin prevents the activation of the IP_3 cascade by ligands (Cockcroft 1987). It has been observed that activation of phospholipase C results from the action of pertussis toxin-sensitive proteins (Cockcroft and Gomperts 1985), whereas in other systems activation is mediated by pertussis-insensitive G-proteins (Martin et al. 1986). On the basis of the ADP-ribosylation and immunochemical experiments it cannot be decided if the G-protein involved in activation of phospholipase C by pheromones is in fact the G_p-protein, as proposed by Cockcroft (1987) for the IP_3-pathway. In a similar approach (ADP-ribosylation) a so-called olfactory GTP-binding protein has been identified in olfactory cilia preparations from vertebrate species (Pace and Lancet 1986).

In view of the persistent lack of direct evidence for specific odorant-receptors in olfactory sensory neurons, the observation that the pheromone-induced stimulation of antennal phospholipase C is mediated by guanine nucleotide regulatory proteins may be considered as a further indication for the existence of specific pheromone-detecting receptor proteins in antennal sensory cells. Insect pheromone receptors could thus be considered as members of the G-protein coupled receptor superfamily (Dohlman et al. 1987). By photoaffinity labelling experiments a candidate polypeptide of pheromone receptors has recently been identified in antennal preparations from male moth (Vogt et al. 1988). The discovery of a G-protein that presumably interacts directly with the putative receptor molecules may help to identify and ultimately isolate pheromone receptor molecules (Novoselov et al. 1988).

Acknowledgements. This work was supported by the Deutsche Forschungsgemeinschaft. A sample of periplanone B was kindly provided by Prof. J. Boeckh. The antisera against G-proteins were a generous gift from Dr. S. Mumby.

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