Video Article

Detection of Phospholipase C Activity in the Brain Homogenate from the Honeybee

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Abstract

The honeybee is a model organism for evaluating complex behaviors and higher brain function, such as learning, memory, and division of labor. The mushroom body (MB) is a higher brain center proposed to be the neural substrate of complex honeybee behaviors. Although previous studies identified genes and proteins that are differentially expressed in the MBs and other brain regions, the activities of the proteins in each region are not yet fully understood. To reveal the functions of these proteins in the brain, pharmacologic analysis is a feasible approach, but it is first necessary to confirm that pharmacologic manipulations indeed alter the protein activity in these brain regions.

We previously identified a higher expression of genes encoding phospholipase C (PLC) in the MBs than in other brain regions, and pharmacologically assessed the involvement of PLC in honeybee behavior. In that study, we biochemically tested two pharmacologic agents and confirmed that they decreased PLC activity in the MBs and other brain regions. Here, we present a detailed description of how to detect PLC activity in honeybee brain homogenate. In this assay system, homogenates derived from different brain regions are reacted with a synthetic fluorogenic substrate, and fluorescence resulting from PLC activity is quantified and compared between brain regions. We also describe our evaluation of the inhibitory effects of certain drugs on PLC activity using the same system. Although this system is likely affected by other endogenous fluorescence compounds and/or the absorbance of the assay components and tissues, the measurement of PLC activity using this system is safer and easier than that using the traditional assay, which requires radiolabeled substrates. The simple procedure and manipulations allow us to examine PLC activity in the brains and other tissues of honeybees involved in different social tasks.

Video Link

The video component of this article can be found at https://www.jove.com/video/58173/

Introduction

The European honeybee (*Apis mellifera* L.) is a eusocial insect, and female bees show caste-dependent reproduction and age-dependent division of labor. For example, in the sterile caste of bees referred to as 'workers', younger individuals feed the broods while older ones forage nectar and pollen outside the hive[1]. Learning and memory ability is critically important in the life of the honeybee, because foragers must repeatedly go back and forth between food sources and their nest and then communicate the locations of good food sources to their nestmates through dance communication[1]. Previous studies demonstrated that the MB, a higher brain center in insects, is involved in the learning and memory ability of the honeybee[2,3,4]. Differentially expressed genes and proteins have been identified in various brain regions of the honeybee[5,6,7,8,9,10,11], suggesting that they are related to the unique functions of each brain region. Although the pharmacologic inhibition or activation of a protein of interest is a well-used approach to reveal the function of the protein in honeybee behavior[12,13,14], it is unknown whether all drugs have functional effects in different regions of the honeybee brain. The validation of the functions of such drugs will strengthen conclusions in studies of behavioral pharmacology.

Here, we focus on PLC, one of the enzymes implicated in mouse cognition[15,16,17,18]. PLC triggers calcium signaling by degrading phosphatidylinositol 4,5-bisphosphate (PIP_2) into inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG)[19,20,21]. IP_3 opens IP_3 receptors on the endoplasmic reticulum (ER), leading to the release of calcium ions from the ER. The released calcium activates both calcium/calmodulin-dependent protein kinase II (CaMKII) with calmodulin and protein kinase C (PKC) in the presence of DAG. Both protein kinases are involved in learning and memory[22,23], consistent with the involvement of PLC in this process. PLCs are categorized into subtypes, including PLCβ, PLCγ, and PLCε, based on their structures[20]. Each PLC subtype is activated in a different context[20], and genes encoding those subtypes are differentially expressed in different tissues. We previously demonstrated that honeybee MBs express genes encoding PLCβ and PLCε subtypes at higher levels than the remaining brain regions[24], and that two pan-PLC inhibitors (edelfosine and neomycin sulfate [neomycin]) decrease PLC activity in different brain regions and, indeed, affect the learning and memory ability of the honeybee[24].

Traditionally, the enzymatic activity of PLC has been measured using radiolabeled PIP_2[25], which requires appropriate training, equipment, and facilities. Recently, a synthetic fluorogenic substrate of PLC has been established[26], making it easy to assess PLC activity in the standard

laboratory. Here, we present a detailed protocol to detect PLC activity in different brain regions of the honeybee using the fluorogenic substrate and to subsequently test the inhibitory effects of edelfosine and neomycin on PLC in these tissues. Because the protocol requires only basic manipulations, it may be applicable to studies of PLC activity in other tissues or brain areas in bees allocated to different social tasks.

Protocol

1. Capture of Foraging Honeybees

1. Purchase honeybee colonies from a local distributor.
2. Using an insect net, catch forager bees which return to the hive with pollen bags on their hind legs. Transfer the bees to a standard 50-mL plastic conical tube and cap the tube (**Figure 1**). Put the tube on ice to anesthetize the bees.
   NOTE: Wear the designated jackets for beekeeping to avoid bee stings. Nursing bees can also be collected depending on the experiment. To catch the nurse bees, observe the bee's behavior on the wax comb and catch nurse bees, which poke their heads into brood cells to feed the larvae, by the wing or thorax using tweezers. Then, place the bees into a 50-mL plastic tube, cap the tube, and put it on ice as mentioned in step 1.2.
3. Capture 12 foragers randomly.
   NOTE: This protocol uses two bees per lot, resulting in six lots. Multiple bees can be caught in one tube, and the number of the bees in a single tube is not important, as bees in each lot are combined later (see step 3.1.1). Keep the tube cool in the summer, as the high temperature in the tube injures the bees.

2. Dissection of the Honeybee Brain

1. In the laboratory, place the 50-mL tube on ice for at least 30 min^{6} to anesthetize the bees.
2. To prepare the dissection stage, fold a piece of dental wax in half (the resulting size is about 3.5 x 7.5 cm) and push it into a plastic dish (60 x 15 mm).
   NOTE: The folded wax firmly holds the insect pins.
3. Under a binocular microscope, separate the bee's head from its body with tweezers and fix it on the dental wax by inserting insect pins at the base of each antenna to hold the anterior of the head in an upward position (**Figure 2A**).
   NOTE: Wash the tweezers before use, using ethanol or sterilized water.
4. Pour enough saline solution (0.13 mol/L NaCl, 5 mmol/L KCl, and 1 mmol/L $CaCl_2$-$2H_2O$) on the head to cover it. Pierce the head again with the pins if the head floats in the solution. Use an ice-cold saline solution, if necessary.
   NOTE: However, this protocol works without the cold saline solution.
5. Remove the antennae using tweezers (**Figure 2B**).
6. Make a horizontal cut near the bases of the antennae, and longitudinally at the border of the compound eyes, using a scalpel. Then, make a horizontal cut at the top of the head. Remove the cuticle to open a window over the brain (**Figure 2C**).
   NOTE: If necessary, wash the scalpel with ethanol or sterilized water before use.
7. Remove the retinae from the ocelli and the tracheae on the anterior surface of the brain, using tweezers (**Figure 2D**).
8. Expand the cut at the border of the compound eyes dorsally and ventrally, using the scalpel (**Figure 2E**). Next, remove the connective tissue between the cuticle of the compound eyes and retinae by inserting the tweezers directly under the eye cuticle (**Figure 2F**).
9. Discard the cuticle of the compound eyes. Pick the dorsal tips of the retinae of the compound eyes with tweezers and move the tweezers in the ventral direction to carefully peel off the retinae (**Figure 2G**).
10. Carefully remove the remaining tracheae on the anterior surface of the brain, using tweezers (**Figure 2H**).
11. Cut the connective tissue between the brain and around the tissues, using tweezers, and dissect the brain from the head capsule (**Figure 2H**).
12. Peel off the tracheae on the posterior surface of the brain, using the tweezers (**Figure 2I**).
13. Using a scalpel, cut the connection between the MBs and other brain regions next to the vertical lobes, which are a part of the MBs (**Figure 2J**).
14. Place the dissected MBs and remaining brain tissues into 1.5-mL tubes and rapidly freeze them with dry ice or liquid nitrogen (**Figure 2K**). Store the frozen tissues at -80 °C until use.
    NOTE: Handle liquid nitrogen with the designated gloves and ventilation to avoid cold burn and suffocation. Dry ice should also be handled carefully with gloves. The protocol can be paused here. The dissection and storage of the brain tissues should be performed one bee at a time to avoid protein degradation.

3. Preparation of Brain Homogenates

1. Add 10 µL of homogenization buffer comprising of 50 mmol/L HEPES-KOH (pH 7.2), 70 mmol/L KCl, and 1.0 mmol/L $CaCl_2$ to the frozen brain tissue and homogenize the tissue in the 1.5-mL tube by manually applying pressure with a plastic pestle. Stroke the tissue at least 200x.
   NOTE: Perform the manipulations described in section 3 on ice. Use a pestle fitting right in the tube to sufficiently smash the tissues, which easily float in the homogenization buffer.
   1. Transfer the homogenized tissue solution to the next tissue in the lot and homogenize the tissue in the same way.
      NOTE: The honeybees in each lot are combined at this step. This protocol uses six lots.
2. Add 30 µL of the homogenization buffer.
3. Centrifuge the homogenized tissue sample for 10 min at approximately 900 x g^{27} and 4 °C.
4. Transfer the supernatant to a new 1.5-mL tube and centrifuge it again for 20 min at approximately 9,500 x g^{27} and 4 °C.
5. Place the supernatant in a new 1.5-mL tube. Store the homogenate at -20 °C until use.

NOTE: The protocol can be paused here.

6. Determine the protein concentration using the bicinchoninic acid (BCA) assay.
    1. Dilute 2.5 µL of the homogenate with 17.5 µL of sterilized water (thus diluting the homogenate eightfold) and use all the diluted homogenate.
    NOTE: Sampling homogenate using a micropipette must be performed carefully because of its high viscosity.
    2. Perform the BCA assay using 0, 0.1, 0.2, 0.4, 1.0, and 2.0 mg/mL of bovine serum albumin (BSA) in 20 µL as standards.
    NOTE: Change the scale of the BCA assay as necessary, based on the volume of the homogenate and standard samples. The protocol can be paused here.

4. PLC Reaction in Brain Homogenates

1. Prepare the stock solution of the fluorogenic substrate WH-15[26] dissolved in sterilized water at 50 µmol/L and store it at -80 °C.
NOTE: Cover the solutions containing the substrate with foil to prevent fading.
2. Prepare the reaction premixture in the required volume to achieve the following composition in 10 µL of reaction mixture: 50 mmol/L HEPES-KOH (pH 7.2), 70 mmol/L KCl, 1.0 mmol/L $CaCl_2$, 2.0 mmol/L dithiothreitol, 50 mg/L BSA, and 12.5 µmol/L WH-15.
3. Dilute the homogenate with homogenization buffer, if required.
4. Mix the reaction premixture and homogenate containing 1.3 µg of proteins in a polymerase chain reaction (PCR) tube to achieve a final volume of 10 µL of reaction mixture.
    1. Prepare two types of control mixtures for the statistical analysis: put the homogenate but not WH-15 into mixture (control mixture 1), and *vice versa* (control mixture 2).
    NOTE: Prepare the reaction mixtures and control mixtures on ice to prevent protein degradation and reaction of PLC. Control mixtures 1 and 2 are used to detect fluorescence from the homogenate and free substrate, respectively.
5. Flush the tube and incubate it in a thermal cycler for 30 min at 25 °C.
6. Stop the reaction by adding 2 µL of 25 mmol/L ethylene glycol bis (β-aminoethylether)-N,N,N',N'-tetraacetic acid.
NOTE: Appropriate protein amounts and reaction times vary among experiments. Thus, to optimize the reaction condition, it might be necessary to repeat the procedures described in steps 4.1 - 5.3 in the preliminary experiments by changing the protein amount and incubation time until the fluorescence increases linearly. For reference, when the homogenate derived from the other brain regions is used, the reaction usually works linearly with 1.3 µg or less of proteins within 30 min, but the linearity decreases either with at least 2.7 µg of proteins or with an incubation time of 90 min or longer.

5. Detection of Fluorescence Resulting from PLC Activity

1. Centrifuge the reaction mixture and control mixtures for 5 min at approximately 310 x *g*[28] and transfer 10 µL of the supernatant to different wells on a 384-well microplate applicable for fluorescence detection.
NOTE: To prevent fading and a contamination with dust, cover the plate with foil.
2. Flush the microplate using a centrifuge for the microplate.
3. Set the plate into a microplate reader and detect fluorescence with a technical triplicate.
NOTE: The excitation and emission wavelengths are 344 nm and 530 nm, respectively. If applicable (depending on the microplate reader), mix the sample mixtures for 5 s before each detection. The protocol can be stopped here.

6. Test of the Inhibitory Action of Pharmacologic Agents

1. Prepare stock solutions of edelfosine and neomycin at the appropriate concentrations and store them until use: edelfosine at 5.0 mmol/L and -20 °C; neomycin at 550 mmol/L and 4 °C.
2. When preparing the reaction premixture as described in step 4.2., add inhibitors to the premixture to achieve the appropriate final concentrations: edelfosine, 1.0 mmol/L; neomycin, 0.55 mmol/L.
3. Add the homogenate to the reaction premixture and appropriate control mixtures as in step 4.4.
    1. Prepare three types of control mixtures: put the homogenate and inhibitor, but not the substrate into control mixture 1; add the substrate and inhibitor but not the homogenate into control mixture 2; and put the inhibitor, but not the homogenate or substrate into control mixture 3.
4. Perform the PLC reaction and detection of fluorescence as described in steps 4.5 - 5.3.

7. Statistical Analysis

1. For the detection of PLC activity using the mixtures described in section 4, calculate the fluorescence derived from the reaction between PLC and the substrate by subtracting the fluorescence emitted from the homogenate or free substrate as follows.
FI (PLC activity) = FI (reaction mix) - {FI (ctrl 1) + FI (ctrl 2)}
NOTE: FI (PLC activity), FI (reaction mix), FI (ctrl 1), and FI (ctrl 2) denote *bona fide* fluorescence derived from PLC activity, fluorescence detected in the reaction mixture, and control mixtures 1 and 2, respectively.
    1. After the measurement of fluorescence in the mixtures as described in steps 4.1 - 5.3, calculate FI (PLC activity) according to the equation in step 7.1, and then the mean FI (PLC activity) of the technical triplicate for each homogenate.
    2. Using the mean FI (PLC activity) of the technical triplicate obtained in step 7.1.1, calculate again the mean FI (PLC activity) of biological replicates of the MBs and other brain regions and compare the values between the brain tissues.

2. For the examination of PLC activity in the presence of inhibitors using the mixtures described in section 6, calculate the fluorescence derived from the reaction among the homogenate, substrate, and inhibitor as follows.
   FI (PLC activity with inhibitor) = FI (reaction mix) - {FI (ctrl 1) + FI (ctrl 2)} + FI (ctrl 3)
   NOTE: Here, FI (PLC activity with inhibitor) is *bona fide* fluorescence resulting from PLC activity in the presence of the inhibitor. FI (reaction mix), FI (ctrl 1), FI (ctrl 2), and FI (ctrl 3) are fluorescence signals detected in the reaction mixture, control mixture 1, control mixture 2, and control mixture 3, respectively.
   1. After the measurement of fluorescence in the mixtures as described in steps 6.1 - 6.4, calculate FI (PLC activity with inhibitor) according to the equation in step 7.2. Then, obtain the mean FI (PLC activity with inhibitor) of the technical triplicate for each homogenate.
   2. Using the mean value calculated in step 7.2.1, calculate the mean FI (PLC activity with inhibitor) of biological replicates derived from each brain tissue. Compare the FI (PLC activity with inhibitor) and the FI (PLC activity) obtained in step 7.1.2 in each brain tissue.

Representative Results

Protein Concentrations in Brain Homogenates:
We prepared homogenates using forager bees. The calculated protein concentrations in the original homogenates are shown in **Figure 3**. The approximate protein concentrations in the original homogenate were as follows: 1.5 mg/mL in the MBs and 2.3 mg/mL in other brain regions. We used two bees per lot and six lots were analyzed.

Detection of PLC Activity in the Brain Homogenates:
In the pilot experiment, we detected higher fluorescence in other brain regions than in the MBs. Therefore, we repeated the preliminary experiments using the homogenate of the other brain regions and determined the reaction time (*i.e.*, 30 min) and protein amount (*i.e.*, 1.3 µg). The result of the reaction under these conditions is shown in **Figure 4A**. The reaction mixtures containing both tissue homogenate and the fluorogenic substrate exhibited > 4.2-fold higher fluorescence than the control mixtures containing either the tissue homogenate or the fluorogenic substrate, suggesting that PLC activity was detected in the reaction mixtures. Relative fluorescence was approximately 3.4-fold higher in other brain regions than in the MBs (**Figure 4B**).

Analysis of the Inhibitory Effects of Pharmacologic Agents on PLC Activity:
To examine the effects of the pan-PLC inhibitors edelfosine and neomycin on PLC activity, we performed the reaction in the presence of 1.0 mmol/L edelfosine or 0.55 mmol/L neomycin, using the same homogenate as analyzed above. In the presence of edelfosine, the fluorescence level decreased to approximately 6.0% and 5.4% in the MBs and other brain regions, respectively, compared to controls without edelfosine (**Figure 4C**). Neomycin treatment reduced the fluorescence level to 44% and 20% that of untreated controls in the MBs and other brain regions, respectively (**Figure 4D**).

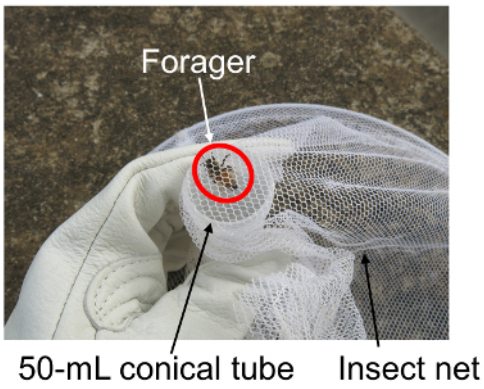

Figure 1: Capturing a forager honeybee. A forager returning to her hive was captured in an insect net, and she was confined into a 50-mL plastic conical tube. Please click here to view a larger version of this figure.

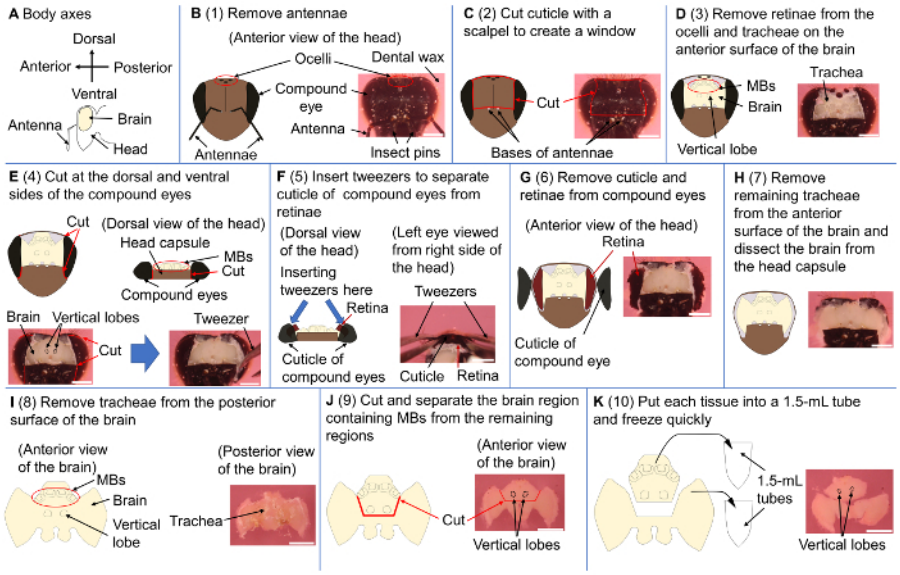

Figure 2: Schematic representation of the dissection procedure. (**A**) Axes of the honeybee brain mentioned in the protocol are shown. The bee from the head to the anterior thorax is viewed from the lateral side. (**B** - **K**) Photos and illustrations of the dissection procedures are shown. See the main text for details. Only the head of the honeybee is presented. An illustration of the tracheae is omitted. MBs = mushroom bodies. The scale bars correspond to 1 mm. Please click here to view a larger version of this figure.

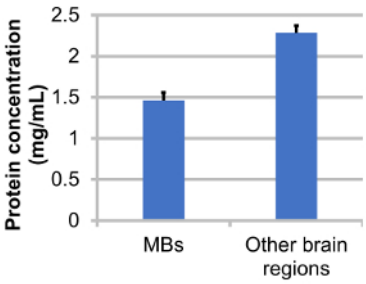

Figure 3: Protein concentrations in brain tissue homogenates. Protein concentrations in the original homogenates were measured by BCA assay and calculated. The mean concentrations with standard deviations are shown. Two forager bees were used for each lot, and six lots were analyzed. MBs = mushroom bodies. Please click here to view a larger version of this figure.

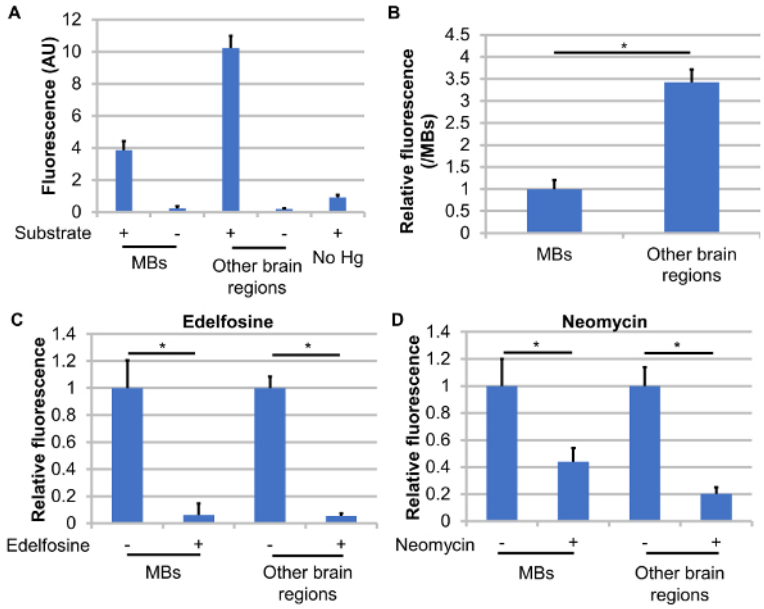

Figure 4: Fluorescence detected in reactions. (**A**) This panel shows fluorescence in each tissue with or without the fluorogenic substrate. The reaction was performed for 30 min using 1.3 µg of protein. Fluorescence was measured by the microplate reader. The values of the sample wells were corrected by empty wells and shown in arbitrary units (AUs). (**B**) This panel shows a comparison of fluorescence between brain tissues. The data in panel **A** were corrected for control mixtures by subtraction and normalized by the calculated fluorescence in the MBs. * *P* < 0.005, Mann-Whitney's U test. Panels **C** and **D** show relative fluorescence in the presence of (**C**) 1.0 mmol/L edelfosine and (**D**) 0.55 mmol/L neomycin. The same homogenates used in panels **A** and **B** were analyzed. The fluorescence values were normalized by the results of the control experiment without drug treatment for each tissue. The data of the control reactions in panel **C** are the same as in panel **B**. In panel **D**, all homogenates were analyzed again in a different experiment. The mean fluorescence values with standard deviations are shown. Two bees were used for each lot, and six lots were analyzed. * *P* < 0.05, Wilcoxon signed-ranks test. No Hg = control mixture not containing homogenate; MBs = mushroom bodies. Panels **B** - **D** were modified from Suenami *et al.*[24] with the publisher's permission. Please click here to view a larger version of this figure.

Discussion

The biochemical examination of protein activity is profoundly important for understanding molecular signaling in the brain, because the activity of an enzyme is affected by various molecules, such as substrates and inhibitors, and can, thus, change along with animal behavior (*e.g.*, learning and memory)[5]. In honeybee studies, enzymes such as cyclic AMP-dependent protein kinase A, cyclic GMP-dependent protein kinase, PKC, phosphorylated CaMKII, and adenylate cyclase are reported to be differentially expressed in various brain regions based on immunohistochemistry[5,10,29,30,31]. Differences in enzymatic activity among brain regions, however, are only partially reported[6]. Here, we described a detailed protocol for detecting PLC activity and assessing the inhibitory effects of pharmacologic agents in the MBs and other brain regions.

There are some possible factors interfering with fluorescence detection. First, it is important to protect the protein from degradation when performing biochemical assays. In the protocol described here, a quick dissection and freezing of the brain are required. It is recommended that tissue samples are frozen and stored soon after each cycle of dissection. A minimization of the thaw/freeze cycle of the homogenate is also crucial to prevent the deterioration of the protein.

In addition to protein degradation, background fluorescence and absorbance in the reaction mixture may affect the results in this assay system, because PLC activity is detected by a fluorescence signal. For example, neomycin dissolved in water has a yellow color that affects fluorescence detection. Although we used 0.55 mmol/L neomycin, a 1000-fold dilution of the stock solution, a further optimization of the concentration might be required. Moreover, contamination by other tissues may also influence the result. The retina, which detects visual stimuli and contains pigments, comprises one tissue type that potentially interferes with the assay.

With the present protocol, we detected higher PLC activity in other brain regions than in the MBs[24]. This was inconsistent with the result of quantitative reverse-transcription PCR analysis, which revealed that the MBs express higher levels of PLC genes than other brain tissues[24]. This contradiction might be due to WH-15, which is a free-floating substrate[26], and the fact that we did not distinguish the membrane and cytosolic fractions of the brain homogenates. Considering that PLCβ and PLCε are membrane-associated enzymes[32] and can interact with the endogenous PIP_2 substrate, the amount of the membrane fraction in the homogenate likely affects the reaction between PLC and WH-15. Another possible explanation is that the PLC concentration might be higher in the other brain regions than in the MBs due to differences in protein production and/or degradation rates. Hence, *bona fide* PLC activity in the brain must be clarified further by additional experiments, such as by using a recently reported new substrate incorporated into the membrane[33], analyzing the activity of membrane-associated and cytosolic PLCs separately, or quantifying the content of PLCs or PIP_2 in each brain tissue.

Taking the above points into account, the PLC assay system described here can be expanded to measure PLC activity in different tissues not assessed here, such as the digestive tract, muscle, and reproductive organ. It is also possible to compare PLC activity between the brains of forager and nurse bees to evaluate the involvement of PLC activity in different social roles.

Overall, the assay system presented here is a feasible option for detecting PLC activity in tissue homogenates, because it can be performed with standard laboratory equipment, while a traditional approach using radiolabeled PIP_2 requires specialized facilities, training, and equipment for radioisotopes. Taking advantage of the system with further modifications will deepen our understanding of the molecular mechanisms underlying the honeybee's complex behavior.

Disclosures

The authors have nothing to disclose.

Acknowledgements

Figure 4B - **4D** was modified from Suenami *et al.*[24] with the permission of Biology Open. The authors are grateful to the publisher for the permission. This work was supported by the Human Frontier Science Program (RGY0077/2016) to Shota Suenami and Ryo Miyazaki.

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