

Measuring the Binding of Protein Kinase C to Sucrose-Loaded Vesicles

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1. Introduction

The binding of protein kinase C (PKC) to model membranes provides useful information about the mechanisms dictating the translocation of PKC to biological membranes. The sucrose-loaded vesicle (SLV) assay is useful for this purpose. It was originally designed to measure the binding of phospholipase C to vesicles (**1**) but later modified to be useful for the study of PKC (**2–4**). Large unilamellar vesicles filled with sucrose are used as model membranes that can be readily pelleted by low-speed centrifugation (*see Subheading 3.1.*). PKC is incubated with the vesicles and other cofactors required for binding, and the mixture is centrifuged to pellet the vesicles along with any PKC bound to them. The supernatant containing the unbound PKC is separated from the vesicle pellet containing bound PKC (*see Subheadings 3.2. and 3.3.*). The relative amounts of enzyme in the supernatant and pellet are determined by a kinase assay (*see Subheading 3.4.*). The percentage of PKC bound can be calculated from these values (*see Subheadings 3.5. and 3.6.*). This assay is useful for determining various properties of PKC. For example, different compositions of lipids can be used to make the SLVs and the binding of PKC to each of them can be compared, to determine which lipids or combinations of lipids are preferred by the enzyme as well as optimal conditions for binding. Also, the binding of mutant PKCs can be compared to that of wild-type to determine the differences created by mutating specific residues.

2. Materials

2.1. SLVs

1. Lipids can be purchased from Avanti Polar Lipids.
2. 1-Palmitoyl, 2-oleoyl-phosphatidylserine (POPS) in chloroform (store at -20°C under argon or nitrogen).
3. 1-Palmitoyl, 2-oleoyl-phosphatidylcholine (POPC) in chloroform (store at -20°C under argon or nitrogen).
4. 2:1 (v:v) chloroform:methanol (store at room temperature in dark).
5. [^3H]1,2-dipalmitoylphosphatidylcholine (DPPC), 60 Ci/mmol, 1 $\mu\text{Ci}/\mu\text{L}$ (store at -20°C).
6. 1 mM phorbol myristate acetate (PMA) in dimethyl sulfoxide. Caution: PMA is a carcinogen (store at -20°C in dark).
7. Sucrose buffer: 170 mM sucrose, 5 mM MgCl_2 , 20 mM HEPES, pH 7.5. Store at 4°C .
8. KCl buffer: 100 mM KCl, 20 mM HEPES, 5 mM MgCl_2 .
9. Microextruder from Avanti or Avestin.
10. Polycarbonate membranes (100-nM pore size).

2.2. PKC Dilution

1. Purified PKC, stored at -80°C .
2. 1 M dithiothreitol (DTT).
3. 1 M HEPES, pH 7.5.
4. 3 mg/mL bovine serum albumin (BSA).

2.3. Binding

1. Binding buffer: 0.36 mg/mL BSA, 120 mM KCl, 240 μM CaCl_2 , 6 mM MgCl_2 , 24 mM HEPES, pH 7.5, 1.2 mM DTT.
2. Buffer A: 0.3 mg/mL BSA, 100 mM KCl, 200 μM CaCl_2 , 5 mM MgCl_2 , 20 mM HEPES, 1 mM DTT.
3. Buffer B: 0.5 mg/mL BSA, 167 mM KCl, 333 μM Ca^{2+} , 33 mM HEPES, 8.3 mM MgCl_2 , 1.7 mM DTT.

All three are stable for several weeks at 4°C .

2.4. Kinase Assay to Determine the Amount of PKC in Supernatant and Pellet

1. "GO": 25 mM MgCl_2 , 20 mM HEPES, pH 7.5, 500 μM cold ATP, 100 μCi $\gamma\text{-}^{32}\text{P}$ ATP, 1 mg/mL protamine sulfate.
2. "STOP": 0.1 M ATP, 0.1 M EDTA, pH 8.0.
3. Whatman P-81 ion exchange paper.

3. Methods

Methods will be described using specific volumes and concentrations for a standard assay, but these can be varied as needed (variations will be described

in **Subheading 4.**) This section will describe how to make SLVs from pure lipid (**Subheading 3.1.**), how to dilute an aliquot of PKC for use in the binding assay (**Subheading 3.2.**), how to bind PKC to the vesicles and separate the bound from the unbound form (**Subheading 3.3.**), how to assay both the bound and unbound fractions to determine the amount of PKC in each of them (**Subheading 3.4.**), how to determine the percentage of SLVs that sediment (**Subheading 3.5.**), and finally how to calculate the fraction of PKC bound (**Subheading 3.6.**).

3.1. Making SLVs

This section will describe how to prepare the SLVs for use in **Subheading 3.3.**

1. Dilute POPS and POPC lipid solutions to 12.5 mM in 2:1 (v/v) chloroform:methanol. (If purchased in powder form, these can be weighed out and dissolved.)
2. Add 80 μL of 12.5 mM POPS and 120 μL of 12.5 mM POPC to a glass test tube.
3. Add 1 μL of [^3H]DPPC to test tube.
4. Vortex well.
5. Evaporate chloroform:methanol under a stream of nitrogen gas to leave a thin film of lipid around the bottom of the tube.
6. Dry under vacuum for 2 h.
7. Add 500 μL of sucrose buffer.
8. Vortex well.
9. Seal tube with Parafilm, but poke a hole in it.
10. Hold the tube with long tweezers, and place the portion of tube containing sucrose in liquid nitrogen, shaking gently. Tube will make a hissing noise, which indicates the solution inside has frozen.
11. Thaw under hot water.
12. Dry the outside of the tube completely and repeat the freeze-thaw cycle four times.
13. Extrude 300 μL of lipid using a microextruder (follow directions from manufacturer for assembly).
14. Use two 100-nm pore-size polycarbonate filters for extrusion.
15. Pass about 200 μL of sucrose buffer through extruder to wet the inside and ensure there are no leaks.
16. Discard, and then load vesicles.
17. Twenty full passes (back and forth) of the lipid through the filters should produce vesicles of uniform size. Remove vesicles from the opposite side to which they were added to ensure all have passed through the filter at least once.
18. Lipid solution becomes clearer after extrusion but remains slightly turbid.
19. Place 250 μL of extruded vesicles in a 1.5-mL ultracentrifuge tube.

20. Add 800 μL of KCl buffer.
21. Centrifuge at 100,000 g for 30 min at 25°C (100°K; 50,000 rpm in a Beckman ultracentrifuge model TLA 120 rotor).
22. Remove 800 μL of KCl buffer.
23. Seal centrifuge tube with parafilm and vortex remaining lipid well (it should become slightly cloudier).
24. Add 5 μL of extruded and centrifuged lipid to 2 mL of scintillation fluid.
25. Add 5 μL of unextruded lipid to 2 mL of scintillation fluid.
26. Incubate both overnight since ^3H counts tend to increase slightly over the first few hours.
27. Determine counts per minute.
28. Use ratio of counts before and after extrusion to determine concentration of extruded vesicles.
29. Use KCl buffer to dilute extruded vesicles to 1 mM. The original vesicles were 5 mM, and are typically 3–4 mM after extrusion and sedimentation. There will be much more than needed for the one binding assay described below, but usually more than one assay is done at once.

3.2. PKC Dilution

This section will describe how to dilute the PKC for use in the binding assay. The amount of PKC used for each assay will vary slightly depending on the PKC preparation, the isoform used, and the specific activity of the enzyme. Typically, about 150–200 ng of PKC is used per assay tube. Because PKC obeys the law of mass action, the absolute amount of enzyme used will not change the fraction of it that binds.

Sample dilution:

- 2 μL of purified PKC (about 150–200 ng).
- 3 μL of 1 M DTT.
- 3 μL of 3 mg/mL BSA.
- 0.6 μL of 1 M HEPES.
- 21.4 μL of H₂O.

The total volume to be added to each tube is 30 μL (*see Subheading 3.3., step 6*).

3.3. Binding Assay

This section will describe the binding of PKC to the SLVs and separation of bound from unbound PKC; binding to isolated domains of PKC can also be measured (*see Note 1, Fig. 1*).

1. Add 4 μL of 1mM PMA to 200 μL of 1mM extruded vesicles. PMA should be added to the side of the Eppendorf tube, and vortexed vigorously for 30 s. If added directly to the vesicles, it may come out of solution.

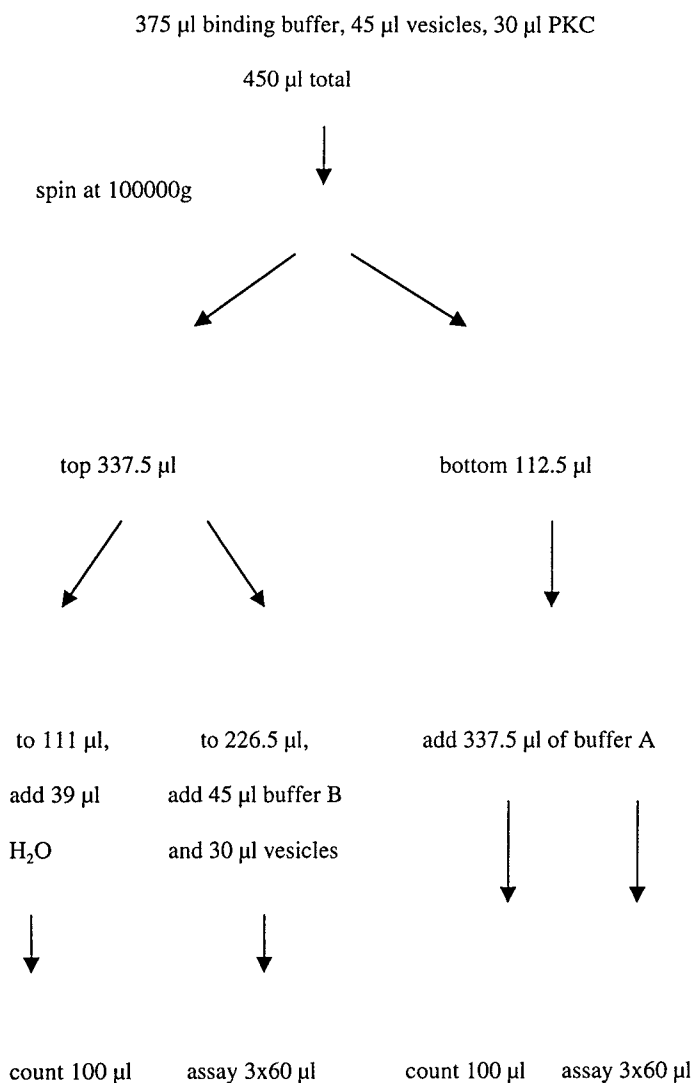


Fig. 1. Schematic of binding assay.

2. Incubate the PMA and vesicles at room temperature for at least 30 min, vortexing occasionally. These vesicles contain 2 mol% PMA.
3. Add 375 μ L of 1.2X binding buffer to each of two centrifuge tubes (*see Note 2*).
4. Add 45 μ L of vesicles to one tube.
5. Add 45 μ L of KCl buffer to the other tube. This tube is a control, and referred to as the “no lipid” tube. Always substitute KCl buffer in this tube whenever lipid is called for.

6. Add 30 μL of diluted PKC to each tube.
7. Mix well with a pipet.
8. Incubate for 10 min at room temperature.
9. Centrifuge at 100,000g for 30 min at 25°C.
10. Remove 337.5 μL of supernatant and store it in an Eppendorf tube. Label this the “kinase tube.”
11. Add 337.5 μL of buffer A to pellet fraction, parafilm, and vortex well. This sample will be used in the kinase assay below, as well as for counting the amount of ^3H in the sample.
12. Remove 111 μL from the “kinase tube” and add it to 39 μL of H_2O . Save this for counting ^3H (do not use for kinase assay below).
13. Add 45 μL of buffer B and 30 μL of vesicles to remaining 226 μL of supernatant in the “kinase tube.”
14. Substitute KCl buffer for vesicles for the “no vesicles” tube.
15. Mix all tubes well.
16. Add 60 μL from the “kinase tube” in **step 13** to each of three plastic culture tubes (to obtain results in triplicate).
17. Add 60 μL of pellet fractions from **step 11** to each of three plastic culture tubes (save the remainder of the fraction for counting ^3H).
18. Do the same for the “no lipid” controls.
19. Add a set of three “blanks” containing only 60 μL of buffer A and no PKC.

3.4. Kinase Assay

This section will describe the kinase assay, which is used to determine the amount of PKC in supernatant and pellet fractions. The activity obtained for each fraction is proportional to the amount of enzyme present (*see Note 3*).

This assay is timed, so prepare everything before starting (*see Note 4*).

1. Add 15 μL of “GO” solution to each tube.
2. Vortex quickly and incubate at 30°C for 6 min.
3. Add 25 μL of “STOP” solution to each tube, in the same order that the “GO” solution was added.
4. Mix briefly and place on ice.
5. Add 80 μL of each reaction to a numbered 2-cm² piece of P-81 ion exchange paper. The positively charged protamine sulfate will bind to the papers.
6. Let all papers dry.
7. Wash for 5 min in 200 mL of 0.4% phosphoric acid. This step washes away all of the ATP that is nonspecifically bound to the ion exchange papers, while leaving the protamine sulfate.
8. Remove papers then dispose of radioactive phosphoric acid appropriately.
9. Repeat **steps 7** and **8** three times.
10. Use 200 mL of ethanol for the fifth wash.
11. Remove papers and separate into scintillation vials.

12. Determine the counts per minute for each paper in a scintillation counter set to count ^{32}P (see **Note 5**).

3.5. Determination of Percentage of Vesicles Sedimented

The amount of ^3H in each supernatant and pellet fraction is counted, to determine the percentage of vesicles that have sedimented (see **Note 6**).

1. Add 100 μL of supernatant fractions (from **Subheading 3.3., step 12**) to 2 mL of scintillation fluid.
2. Add 100 μL of pellet fractions (from **Subheading 3.3., step 17**) to 2 mL of scintillation fluid.
3. Determine counts per minute in a scintillation counter set to count ^3H .

3.6. Calculations

This section will explain the calculations used to determine the fraction of PKC bound to the SLVs. From the amount of bound PKC, one can then calculate the apparent membrane affinity.

First, the percentage of vesicle associated activity (A_v) is determined using **Eq. 1**:

$$A_v = \frac{\beta A_b + (\beta - 1) A_t}{\alpha + \beta - 1} \quad (1)$$

where A_b and A_t are the measured activities of the bottom and top fractions, respectively. α is the fraction of sedimented vesicles and is calculated from the distribution of ^3H -labeled PC between the bottom and top fractions. β is fraction of kinase activity measured in the supernatant in the absence of lipid; within the limits of experimental error, it should be equal to the value expected for a nonsedimenting protein (i.e., 0.75 under the experimental conditions described; see **Note 7**).

Second, the fraction of bound PKC is calculated by taking the ratio of the vesicle-associated activity over the sum of the activities in the top and bottom fractions as in **Eq. 2**:

$$\% \text{ bound} = \frac{A_v}{A_b + A_t} \quad (2)$$

The apparent membrane affinity of the enzyme (apparent K_A) can now be calculated. It is defined as the ratio of membrane-bound to free enzyme divided by the total lipid concentration. Note that for accurate determination of K_A , it is important to measure binding under conditions where approx 50% of the PKC is bound.

4. Notes

1. A variation in the assay is to measure the membrane binding of either C1 or C2 domains of PKC rather than full length enzyme (**4**). In this case, total protein is detected by SDS-PAGE analysis of bound vs free protein, followed by either silver staining or Western blot analysis. The amount of protein used in the binding step needs to be increased slightly so that it is enough to see on a gel but keeping the vesicle lipid in large excess of the protein. If using either a silver stain or a Western to determine the amount of PKC in the supernatant and pellet, simply separate the 337 μL of supernatant from the 112 μL of pellet, and then run one-third of each sample on a gel. There is no need to go through **steps 11–19** of **Subheading 3.3**. The gel replaces **Subheading 3.4**. (there is no need to add vesicles back to the supernatant fraction after separation because the amount of PKC is being determined by silver stain or a Western rather than by kinase activity). The fraction of PKC bound is the same regardless of the method used to determine it.

The amount of PKC in the supernatant and pellet need to be determined by densitometry in the case of a silver stain, or by autoradiography in the case of a Western. Use the absolute amounts determined in place of counts per minute in **Subheading 3.6**. Instead of counting 100 μL of supernatant and pellet in **Subheading 3.5**, count 33.7 μL of supernatant, and 11.2 μL of pellet fraction.

2. The volumes used for the binding steps above can be varied. The important part is to keep all the proportions the same. The rotor in the method above uses centrifuge tubes that hold volumes of up to about 1 mL. It is recommended that reaction volumes be at least 450 μL ; smaller volumes result in small pellets that tend to float up when the supernatant fraction is pipetted off.
3. It is important that the kinase assay is linear over the entire 6-min reaction. This can be tested easily by setting up six reaction tubes and adding the “STOP” at different time intervals up to 6 min. The activity obtained should be linear over the entire 6 min. If not, the amount of PKC used needs to be reduced.
4. The assay described had only six tubes during the kinase portion. If there are many different conditions for an assay, the kinase portion of the procedure will get very cumbersome. It is easier to do the kinase **Subheading 3.4**, **steps 1–4**, in groups of 24 tubes, leaving the remaining tubes on ice.
5. Ideally, the sum of the activity in each supernatant and pellet fraction should be the same under each condition, since the same amount of PKC is added to each centrifuge tube. Realistically, this is not always the case, because there is some variation in the activation *seen* with different lipid compositions and amounts. The important part is that both the supernatant and pellet are treated similarly, in that they have the same amount and composition of vesicles added to them. Therefore, the ratio of the supernatant and pellet will be meaningful.
6. The percentage of vesicles in the pellet (**Subheading 3.6**) should be at least 95% to avoid anomalies in the equation. If values are too low, it is possible that vesicles are being pipetted into the supernatant. Care should be taken not to pipet too close to the bottom of the tube, or to pipet the supernatant up too quickly.

Also, different phospholipids may sediment differently. Standard POPS/POPC vesicles should have about 96–98% in the pellet.

7. Different PKC preparations may have a different value for “no lipid,” depending on whether there is some aggregation/denaturing affecting solubility. Ideally, this should be about 0.75 because this is the fraction of the total volume in the supernatant, but it is sometimes lower if there is some insoluble PKC in the preparation. Values for domains are sometimes lower.

Acknowledgment

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References

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