Measuring cAMP Specific Phosphodiesterase Activity: A Two-step Radioassay

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[Abstract] Cyclic nucleotide degrading phosphodiesterase (PDE) enzymes are crucial to the fine tuning of cAMP signaling responses, playing a pivotal role in regulating the temporal and spatial characteristics of discrete cAMP nanodomains and hence the activity of cAMP-effector proteins. As a consequence of orchestrating cAMP homeostasis, dysfunctional PDE activity plays a central role in disease pathogenesis. This highlights the need for developing methods that can be used to further understand PDE function and assess the effectiveness of potentially novel PDE therapeutics. Here we describe such an approach, where PDE activity is indirectly measured through the direct quantification of radioactively tagged cAMP (pmol/min⁻¹/mg⁻¹). This method provides a highly sensitive tool for investigating PDE functionality.

Keywords: PDE, Phosphodiesterase, cAMP, Cyclic adenosine-monophosphate, 5'-AMP, 5' adenosine monophosphate

[Background] Cyclic 3',5'-adenosine monophosphate (cAMP) is a ubiquitously expressed second messenger, synthesised by adenylate cyclase enzymes (ACs), that acts as a master regulator of a broad spectrum of intracellular signalling pathways (Hayes and Brunton, 1982; Beavo and Brunton, 2002). Homeostatic regulation of cAMP is fine-tuned by phosphodiesterase enzymes (PDEs), rapidly degrading cAMP into 5'AMP (Baillie, 2009). PDEs consist of 11 families (PDE1-11) spanning over 100 isoenzymes (e.g., PDE4A1-PDE4A5), all with the ability to rapidly hydrolyse cyclic nucleotides (Conti and Beavo, 2007). Of these, PDE1-4, 7, 8, 10 and 11 possess the ability to catalyse the hydrolysis of cAMP. Compartmentalisation of specific PDE isoforms within discrete signalosomes is crucial to the spatio-temporal control of cAMP nanodomains, tightly regulating cAMP-effector protein activity (Blair and Baillie, 2019). In light of this, it is no surprise that aberrant PDE activity plays a central role in disease pathogenesis, with many PDEs being identified as a driving force in cardiovascular disease (Bobin *et al.*, 2016), respiratory disease (Zuo *et al.*, 2019), neurological disease (Knott *et al.*, 2017) and cancers (Peng *et al.*, 2018). Thus, research and development into novel and effective therapies targeting PDE activity remains a highly competitive area in drug discovery (Maurice *et al.*, 2014; Baillie *et al.*, 2019). This demonstrates a clear need for the development of assays with the ability to measure PDE activity, not only as a tool to investigate PDE activity/function in cellular signaling, but as a means of assessing and developing therapies targeting PDE activity.

Marchmont and Houslay (1980) previously described a method of measuring PDE activity, modified

from a two-step procedure developed by Thompson and Appleman (1971). This radioassay directly quantifies the formation of radioactively tagged 8-³H adenosine, formed as a result of cAMP hydrolysis by PDE within a given sample, allowing for the rate of cAMP hydrolysis to be determined. This highly sensitive assay has proven to be an invaluable tool in the understanding of unique PDE functionality and in assessing the effectiveness of novel PDE therapies (example studies utilising this method: Schafer *et al.*, 2010; Moretto *et al.*, 2015; Omar *et al.*, 2019; Houslay *et al.*, 2019). Here we present a straight-forward and effective procedure for indirectly measuring the activity of cAMP hydrolysing PDEs (Figure 1).

Figure 1. Simple schematic representing Phosphodiesterase Assay procedure

Materials and Reagents

1. Eppendorf® tubes (1.5 ml, clear tubes) (Greiner Bio-one, catalog number: 616-201)
2. Magnetic stir plate
3. pH paper
4. Tris Base (20 mM, pH 7.4 with HCl) (Fisher Scientific, catalog number: 77-86-1)
5. MgCl₂ (VWR, catalog number: 25108.260)
6. Dowex (Sigma-Aldrich, catalog number: 44340)
7. Absolute Ethanol (VWR, catalog number: 20821.330)
8. Deionized H₂O (Milli-Q Pure)
9. Adenosine 3'5'-cyclic monophosphate, sodium salt (Sigma-Aldrich, catalog number: A6885, storage -20 °C)
10. 8-³H-cAMP substrate (Amersham Biosciences, catalog number: TRK304-1mCi, storage -20 °C)
11. Snake venom (Ophiophagus Hannah) (Sigma, catalog number: V0376, storage -20 °C)
12. Opti-Flow SAFE 1 scintillant (Fisher Scientific (chemicals), catalog number: SC/9255/21, storage: room temperature)
13. NaOH (Fisher Scientific, catalog number: 4920/53)
14. HCl (Fisher Scientific, catalog number: H/1200/PB17)
15. Buffer A (see Recipes)
16. Buffer B (see Recipes)

17. Dowex Anion Exchange Resin (see Recipes)
18. Snake Venom (see Recipes)
19. cAMP Substrate Solution (see Recipes)

Equipment

1. TRI-CARB 2900TR Liquid Scintillation Analyzer (Packard) (Figure 2)
More up-to-date models exist (*e.g.*, TRI-CARB 4910TR 110V Liquid Scintillation Counter, Packard)

Figure 2. TRI-CARB 2900TR Liquid Scintillation Analyzer (Packard)

Software

1. Microsoft Excel

Procedure

1. Activating Dowex 1x8-400 Anion Exchange Resin.
Prepare large batches of Dowex resin:
 - a. Dissolve 400 g Dowex resin in 4 L 1 M NaOH.
 - b. Mix solution at room temperature for 15 min.
Note: To mix, a magnetic stir plate was used.
 - c. Allow resin to settle by gravity and remove supernatant.
 - d. Wash resin with 4 L of deionized H₂O until pH 7.0, using pH paper to measure.
Note: This may take up to 30 x washes.
 - e. At pH 7.0, allow resin to settle and remove supernatant.
 - f. Suspend resin in 4 L 1 M HCl and mix for 15 min at room temperature.
 - g. Allow resin to settle and remove supernatant.

- h. Finally, wash resin in 4 L of deionized H₂O 3x until pH 3.0, using pH paper to measure.
 - i. Stop mixing of solution after each wash to allow resin to settle before carefully removing deionized H₂O and refilling with fresh deionized H₂O.
 - ii. Store resin in deionized H₂O at 4 °C.
 - iii. Add ethanol and deionized H₂O to resin at a 1:1:1 ratio (dowex:ethanol:dH₂O) prior to use in assay.
2. Label triplicate 1.5 ml Eppendorf® tubes per sample for assay (*i.e.*, three technical replicates), including blanks. Incubate on ice.
3. Thaw all stock solutions being used.
4. Heat water-bath to 30 °C.
5. Prepare sample(s) to be assayed.
 - a. Incubate appropriate volume/concentration of PDE containing sample in 50 µl Buffer A (Recipe 1).

Note: Pilot studies should be carried out to assess optimal concentration(s) of PDE containing sample being used. Typical concentration range of purified protein/PDE overexpressing cellular lysates: 1-10 µg/µl in a total volume of 50 µl Buffer A.
 - b. If investigating effect of inhibitor(s), make sample up in 40 µl Buffer A and 10 µl of appropriately diluted inhibitor.
 - c. Add 50 µl of Buffer A only for blanks.
6. Add 50 µl of cAMP substrate solution (Recipe 5) to each PDE containing sample, including blanks, and vortex. Radioactively tagged cAMP in mix will be hydrolyzed to 5'-AMP in samples containing PDEs.
7. Incubate samples in a water bath at 30 °C for 10 min.
8. To terminate reaction (*i.e.*, inactivate PDEs in sample), boil samples at 100 °C for 2 min.
9. Cool samples on ice for 15 min.
10. Add 1 mg/ml snake venom to each sample (*i.e.*, 0.2 mg/ml final concentration), including blanks, and mix.

Note: Snake venom dephosphorylates the 5'-AMP to adenosine, preventing 5'-AMP from recircularising into cAMP.
11. Incubate samples in a water bath at 30 °C for a further 10 min.
12. Add 400 µl Dowex:ethanol:dH₂O mix (aka Dowex anion exchange resin) to each sample and vortex. Ensure Dowex anion exchange resin is thoroughly suspended before adding to sample(s). Dowex anion exchange resin separates negatively charged cAMP from uncharged adenosine.
13. Cool samples on ice for a further 15 min.
14. Vortex samples thoroughly and centrifuge at 10,000 x g for 3 min at 4 °C in a refrigerated bench-top centrifuge.
15. Concurrently, add 1 ml of Opti-Flow SAFE 1 scintillant to freshly labeled 1.5 ml Eppendorf® tubes.
16. Remove 150 µl of clear supernatant from PDE containing reaction samples (see Step 14) and

- add to a appropriate scintillant tube containing 1 ml of Opti-Flow SAFE 1 scintillation fluid.
17. Vortex all samples thoroughly.
 18. Using a TRI-CARB 2900TR Liquid Scintillation Analyzer (Figure 2), measure/count unbound 8-³H]-adenosine. This determines the rate of cAMP hydrolysis and thus PDE activity.
 19. Analyse data (*i.e.*, determine PDE activity. See Tables 1 and 2 for example set up/data analyses.)

Table 1. Example experimental set up

Samples	Buffer	Inhibitor	cAMP Substrate Solution	Snake Venom & Dowex
Positive Control <ul style="list-style-type: none"> PDE containing sample 	+		+	+
Test <ul style="list-style-type: none"> PDE containing sample + inhibitor 	+	+	+	+
Negative [Blank] Control <ul style="list-style-type: none"> No PDEs in sample 	+		+	+

Data analysis

1. Analysing scintillation counter results (see Table 2)
(*Example results: Omar et al., 2019*)
 - a. Correct each sample to the background appropriately using blank reaction samples containing no PDEs.
 - b. Use background corrected counts to determine the initial rate of reaction (*i.e.*, cAMP hydrolyzed/min).
 - c. To express results in cAMP hydrolyzed in pM/min¹/mg¹ protein, pre-determine protein concentration of samples used in assay through a chosen standard method (*e.g.*, Bradford assay, BCA assay, *etc.*).
 - d. Thus, use the following formula to determine the activity of a specific PDE within sample:

$$\text{PDE activity} = 2.61 \times \frac{\text{Average Value} - \text{Blank}}{\text{Average Total Count}} \times 10 \times \frac{1,000}{\mu\text{g protein}}$$

- PDE activity = cAMP hydrolyzed in pM/min¹/mg¹
 - 2.61 stands for the Dowex practical concentration
 - 10 represents the concentration of cAMP (in pM) hydrolyzed in 100 µl in 10 min
- e. To determine the effects of a selected PDE inhibitor, directly compare uninhibited control reactions to those that were inhibited and expressed as a percentage of the uninhibited control.

Table 2. Example Data Set. PDE containing samples contained 5 µg total purified PDE protein in 100 µl sample.

Sample Number (Triplicates)	Sample ID	8- ³ H]-adenosine Count	Average Count	PDE activity (pM/ min ⁻¹ /mg ⁻¹)	PDE activity - Mock (Background subtraction)	% Difference vs. PDE control
	Blank		1000			
	Average Total Count		100000			
1	Mock	1505	1500	26.1		
2		1495				
3		1500				
1	PDE	9500	10000	469.8	443.7	100
2		10000				
3		10500				
1	PDE + PDE Inhibitor	2750	3000	104.4	78.3	17.65
2		3000				
3		3250				

Notes

- ³H₁ (Tritium)
Half Life: 12.3 Years
Specific activity: 3.59E + 14 Bq.g⁻¹

Recipes

- Buffer A
20 mM Tris-HCl (pH 7.4)
Store at 4 °C
- Buffer B
20 mM Tris-HCl (pH 7.4)
10 mM MgCl₂
Store at 4 °C
- Dowex anion exchange resin
Dowex:ethanol:deionized H₂O [1:1:1 ratio]
Store at 4 °C
- Snake venom (10mg/ml stock; derived from Ophiophagus Hannah)
Dilute to 1 mg/ml in Buffer A

Store at -20 °C

5. cAMP substrate solution

2 µl 'cold' cAMP (*i.e.*, unlabeled) [1 mM cAMP]

3 µl 'hot' cAMP (*i.e.*, 8-³H-labeled) [1 µCi/µl]

995 µl Buffer B

Store at -20 °C

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Competing interests

The authors have no competing interests.

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