

Regulation of PP2A_C Carboxylmethylation and Cellular Localisation by Inhibitory Class G-Protein Coupled Receptors in Cardiomyocytes

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Abstract

The enzymatic activity of the type 2A protein phosphatase (PP2A) holoenzyme, a major serine/threonine phosphatase in the heart, is conferred by its catalytic subunit (PP2A_C). PP2A_C activity and subcellular localisation can be regulated by reversible carboxylmethylation of its C-terminal leucine309 (leu309) residue. Previous studies have shown that the stimulation of adenosine type 1 receptors (A1.Rs) induces PP2A_C carboxylmethylation and altered subcellular distribution in adult rat ventricular myocytes (ARVM). In the current study, we show that the enzymatic components that regulate the carboxylmethylation status of PP2A_C, leucine carboxylmethyltransferase-1 (LCMT-1) and phosphatase methyltransferase-1 (PME-1) are abundantly expressed in, and almost entirely localised in the cytoplasm of ARVM. The stimulation of G_i-coupled A1.Rs with N⁶-cyclopentyladenosine (CPA), and of other G_i-coupled receptors such as muscarinic M₂ receptors (stimulated with carbachol) and angiotensin II AT₂ receptors (stimulated with CGP42112) in ARVM, induced PP2A_C carboxylmethylation at leu309 in a concentration-dependent manner. Exposure of ARVM to 10 μM CPA increased the cellular association between PP2A_C and its methyltransferase LCMT-1, but not its esterase PME-1. Stimulation of A1.Rs with 10 μM CPA increased the phosphorylation of protein kinase B at ser473, which was abolished by the PI3K inhibitor LY294002 (20 μM), thereby confirming that PI3K activity is upregulated in response to A1.R stimulation by CPA in ARVM. A1.R-induced PP2A_C translocation to the particulate fraction was abrogated by adenoviral expression of the alpha subunit (Gα_{t1}) coupled to the transducin G-protein coupled receptor. A similar inhibitory effect on A1.R-induced PP2A_C translocation was also seen with LY294002 (20 μM). These data suggest that in ARVM, A1.R-induced PP2A_C translocation to the particulate fraction occurs through a G_iPCR-Gβγ-PI3K mediated intracellular signalling pathway, which may involve elevated PP2A_C carboxylmethylation at leu309.

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Introduction

The type 2A protein phosphatase (PP2A) is a serine/threonine protein phosphatase that is ubiquitously expressed in all eukaryotic cells and may account for up to 1% of total cellular protein [1]. A significant proportion (~30%) of PP2A consists as a scaffold (A) subunit complexed with the catalytic (C) subunit to form PP2A_{A/C} heterodimers [2]. The PP2A_{A/C} heterodimer subunits provide a platform for the binding of a third component, the regulatory B subunit, which facilitates “targeting” of the heterotrimeric holoenzyme towards target substrates [3].

The PP2A catalytic subunit undergoes reversible post-translational phosphorylation and carboxylmethylation, both of which can alter catalytic activity and cellular distribution of PP2A. Phosphorylation of threonine304 and tyrosine307 residues in the carboxyl terminus of the protein is associated with inhibition of PP2A_C activity [4,5]. Carboxylmethylation of PP2A_C occurs at the C-terminal leucine309 (leu309) and is catalysed by leucine carboxylmethyltransferase-1 (LCMT-1), a member of a large methyltransferase family of enzymes that utilise S-adenosyl

methionine (SAM/AdoMet) as a universal methyl donor [6]. The carboxylmethylation of leu309 increases the binding affinity of the PP2A_{A/C} heterodimer for some, but not all, regulatory B subunits, which have been classified into four separate sub families and are encoded by 15 human genes: *PPP2R2/B* (A–D), *PPP2R5/B'* (A–E), *PPP2R3/B''* (A–C) and the *striatins/B'''* (1, 3 and 4). Through alternative gene splicing, several of these genes can generate a number of splice variants, resulting in the expression of 20+ regulatory B subunits [7]. The importance of PP2A_C leu309 carboxylmethylation by LCMT-1 for recruitment of regulatory B subunits to the PP2A_{A/C} heterodimer can be considered a sliding scale, whereby it is an absolute prerequisite for *PPP2R2/B* subunit recruitment by the PP2A_{A/C} heterodimer and progressively less important for the recruitment of *PPP2R5/B'*, *PPP2R3/B''* and the *striatin/B'''* subunits to PP2A_{A/C} [8].

The carboxylmethylation of PP2A_C on leu309 is reversed by the protein phosphatase methyl esterase PME-1 [9], an enzyme found to be associated with an inactive and demethylated pool of PP2A_C subunits [10]. Evidence suggests that PME-1 can displace the two metal ions from the active site that are required for PP2A_C activity,

thereby inhibiting PP2A_C activity in a demethylation-independent manner (6). This inactive pool of PP2A_C can also be reactivated by a phosphotyrosyl phosphatase activator (PTPA) *in vitro* [10]. The mechanism of PP2A_C reactivation by PTPA remains undefined, however, evidence suggests that the PTPA-mediated peptidyl-prolyl *cis/trans* isomerase activity and consequent conformational change to the structure of PP2A_C seems likely [11].

Structural X-ray crystallographic studies [12–14] have shown that the last 6 C-terminal amino acids (304TPDYFL309) of PP2A_C interact with both the active site and a unique lid domain of LCMT-1, the latter of which may confer some degree of substrate specificity. These 6 C-terminal amino acids of PP2A_C are highly conserved between species [15] and occupy the deep active site pocket of LCMT-1, which is facilitated by interaction between the catalytic sites of active PP2A_C and LCMT-1, thereby suggesting that PP2A_C can only be carboxymethylated by LCMT-1 once in an active conformation [16]. This ensures that only active PP2A_C subunits can be carboxymethylated and is therefore thought to prevent uncontrolled PPP2R2/B-PP2A-mediated dephosphorylation [16].

The importance of understanding the regulation of cellular PP2A_C activity is confirmed by the number of human diseases such as diabetes [17], cancer [18], Alzheimer's disease [19] and heart failure [20,21] which appear to share (in part) a PP2A-based aetiology. Despite this, the role and regulation of PP2A_C carboxymethylation in cells is poorly understood. In the heart, several studies [22–25] demonstrate that stimulation of adenosine type 1 receptors can induce not only PP2A_C carboxymethylation but also PP2A_C translocation to the membrane-rich particulate fraction of ventricular myocytes. Furthermore, previous work by ourselves [23] and others [22] has shown that the phosphorylation status of selected proteins that are expressed within the membrane-rich particulate fraction of ventricular myocytes is reduced, thereby altering the function of these proteins. Since type 2A protein phosphatases represent the only identifiable LCMT-1 substrates [26], it is surprising that this important LCMT-1/PME-1/PP2A_C intracellular signalling axis has not been more extensively studied in cardiac cells.

Hence, the current study investigates the intracellular signalling mechanisms through which G_iPCR stimulation regulates PP2A_C carboxymethylation and subcellular distribution in ARVM. We demonstrate that (i) the LCMT-1/PME-1/PP2A_C/B55 α intracellular signalling axis is mainly localised in the cytoplasm, (ii) PP2A_C carboxymethylation can be induced by multiple G_iPCR agonists, (iii) A1.R stimulation increases the cellular association between PP2A_C and LCMT-1 and (iv) A1.R-induced PP2A_C translocation to a membrane-rich particulate fraction occurs through a G $\beta\gamma$ -PI3K pathway.

Materials and Methods

Animal tissue used in this study was obtained in accordance with the UK Home Office Guidance on the Operation of the Animals (Scientific Procedures) Act 1986, published by Her Majesty's Stationery Office, London, and approved by the Institutional Animal Care and Use Committee (IACUC) of King's College London. Healthy animals were sacrificed by a schedule one procedure completed by a home office licensed individual such that animal suffering was categorised as minimal.

Antibodies and reagents

Antibodies were from the following sources: monoclonal anti-PP2A_{C α/β} (05-421), monoclonal anti-methyl PP2A_C (2A10) and monoclonal anti-demethylated PP2A_C (4B7) antibodies were

purchased from Millipore/Upstate Biotechnologies, UK; polyclonal anti-G α_{t1} (K20), monoclonal anti-LCMT-1 (4A4), polyclonal anti-PP2A_C (FL-309) and monoclonal anti-PME-1 (B12) antibodies were purchased from Santa Cruz Biotechnology; polyclonal anti-cardiac troponin I (cTnI), monoclonal anti-PKB (2H10), rabbit monoclonal anti-PKB (11E7) and phosphorylated PKB (ser473) were obtained from Cell Signaling Technology. Polyclonal anti-GFP antibody was purchased from Clontech; anti-B55 α (PPP2R2A) polyclonal antibody was purchased from Merck Calbiochem; HRP-conjugated donkey anti-rabbit and sheep anti-mouse secondary antibodies were purchased from GE Healthcare, UK. G_iPCR agonists N⁶-cyclopentyladenosine (CPA), carbachol (CCH) and CGP42112 (CGP) were purchased from Sigma-Aldrich; LY294002 was purchased from Merck Calbiochem. Recombinant adenovirus coexpressing enhanced green fluorescent protein (EGFP) and the G α subunit of transducin (G α_{t1}) was a kind gift from Professor Thomas Wieland [27], University of Heidelberg, Germany.

Cell culture of adult rat ventricular myocytes

ARVM were isolated from the hearts of adult male Wistar rats (200–250 g, B & K Universal Ltd) by collagenase-based enzymatic digestion, as previously described [28]. The isolated myocytes were pelleted by brief centrifugation at 50 *g* and washed at room temperature with M199 culture medium (Invitrogen) containing 100 IU/ml penicillin/streptomycin and 10% FBS. Following further centrifugation at 50 *g*, ARVM were resuspended in modified M199 containing (in mM) creatine 2, carnitine 2, taurine 5, 100 IU/ml penicillin/streptomycin and 10% FBS. ARVM were then added to 6-well cell culture plates, that had been earlier coated with laminin as previously described [28] and allowed to adhere for 90 minutes in an incubator (37°C, 5% CO₂). The culture medium was then replaced with fresh modified M199 medium, prior to adenoviral infection.

Adenoviral infection of adult rat ventricular myocytes

The recombinant adenovirus which co-expresses enhanced green fluorescent protein (EGFP) with the bovine Galpha subunit of the transducin GPCR (AdV:G α_{t1}) was constructed using the AdEasy system [29] and a kind gift from Professor T Wieland, University of Heidelberg [27,30,31]. Adenovirus expressing EGFP alone (AdV:EGFP) was used as a control. The recombinant adenoviruses were amplified in HEK-293 cells and purified over CsCl₂ gradients as previously described [28], which produced high-titre viral stocks of 3–5 $\times 10^9$ plaque forming units (pfu)/ml, as determined by a serial end-point dilution assay [32]. ARVM were infected with AdV:EGFP (control) or AdV:G α_{t1} at a multiplicity of infection (MOI) of 50 pfu/ARVM (which provided >90% transduction efficiency). The adenovirus-containing medium was then removed after 60 min and replaced with fresh modified M199 medium. ARVM were maintained in an incubator (37°C, 5%CO₂) and were used for experiments 18 h after infection.

Stimulation of adult rat ventricular myocytes with G_iPCR agonists

ARVM were exposed to the angiotensin II type 2 receptor agonist CGP42112 (0.01–100 nM, 10 min), the muscarinic receptor agonist carbachol (0.01–100 μ M, 10 min) or adenosine type 1 receptor (A1.R) agonist CPA (0.01–100 μ M, 10 min), to stimulate their cognate GPCRs. Control cells received vehicle (phosphate buffered saline or 0.1% DMSO in PBS) for an identical period. At the end of the exposure period, ARVM were lysed with

either Laemmli SDS-PAGE sample buffer (for immunoblotting) or a lysis buffer for subcellular fractionation.

Immunoprecipitation of PP2A_{C α / β} from adult rat ventricular myocytes

ARVM were exposed to vehicle control (DMSO, 0.1%) or CPA (10 μ M) for 10 min and then lysed with ice-cold immunoprecipitation buffer (in mM); imidazole-HCl 20, EDTA 2, EGTA 2 and 0.1% Triton X-100. The culture plates were frozen on a volume of liquid N₂ and subsequently thawed at room temperature at which point cellular lysates were harvested. The insoluble cellular components were collected as a pellet following centrifugation at 14000 *g* for 10 min at 4°C. A 100 μ l aliquot of the supernatant was then removed and to this 50 μ l of 3 \times Laemmli sample buffer was added, this is referred to as the “input”. To immunoprecipitate PP2A_{C α / β} , 100 μ g of protein from the remaining supernatant was incubated with 5 μ g of immunoprecipitating antibody (mouse monoclonal anti-PP2A_{C α / β}) or non-immune mouse IgG (Millipore, UK), overnight at 4°C. Then 50 μ l of protein A sepharose beads (GE Healthcare) was added for a further 1.5 hours at 4°C. The beads were collected by centrifugation at 370 *g* for 1 min and at 4°C. The immunocomplex was then washed twice with ice-cold PBS and 50 μ l of 2 \times Laemmli sample buffer was added to the immunocomplex. All samples were then heated to 95°C for 5 min prior to SDS-PAGE and Western immunoblotting. Equal volumes (20 μ l) of each sample were loaded per well.

Subcellular fractionation of adult rat ventricular myocytes

ARVM were fractionated using a previously described protocol [33], with minor modifications. In brief, ARVM were lysed in ice-cold lysis buffer at pH 7.5 containing (in mM) Tris-HCl 50, EGTA 5, EDTA 2, DTT 5, as well as 0.05% digitonin and Mini-Complete protease inhibitor cocktail (Roche, Germany). The samples were then frozen by floating the culture plate on a volume of liquid N₂ and subsequently thawed at room temperature, at which point cellular lysates were harvested. Cell lysates were then centrifuged at 14000 *g* for 30 min at 4°C and the supernatant, which contained the cytoplasmic fraction, was removed. The pellet, which contained the membrane-rich particulate fraction, was then solubilised in an equal volume of the digitonin-based lysis buffer containing 1% Triton X-100. The determination of total PP2A_C content in the cytosolic and particulate subcellular fractions was achieved by NaOH-mediated saponification of the methylated C-terminal leu309 residue of PP2A_C. In brief, an equal volume of 200 mM NaOH (100 mM final [NaOH]) was added to each cytosolic and particulate fraction, followed by incubation for 30 min at 30°C and subsequent pH neutralisation by the addition of 1 M HCl, as previously described [23]. This ensured that all PP2A_C protein in the sample was in a demethylated state, thereby allowing detection of total PP2A_C content by the anti-PP2A_C monoclonal antibody (4B7). Equal volumes of Laemmli SDS-PAGE sample buffer were added to both fractions and the fractionated proteins resolved by 12% SDS-PAGE followed by western immunoblotting.

Western immunoblotting

Western immunoblotting was carried out as previously described [34]. In brief, protein samples were separated by 12% SDS-PAGE, transferred to PVDF or nitrocellulose membranes where appropriate and probed with the primary antibodies as described earlier. Primary antibodies were detected by appropriate donkey anti-rabbit or sheep anti-mouse secondary antibodies linked to horseradish peroxidase (GE Healthcare, UK). Specific

protein bands were detected by enhanced chemiluminescence (GE Healthcare, UK) and band intensity was quantified using a calibrated densitometer (GS-800, Bio-Rad) and Quantity One® 1-D analysis software (v 4.6.2).

Statistical analysis

Data are presented as mean \pm SEM. Data were subjected to ANOVA (GraphPad Prism v6.0.1) and if a significant difference ($p < 0.05$) was detected, further analysis by a Dunnett’s modified t-test (for comparison of each group with a single control) was performed.

Results

Compartmentalisation of the PP2A_C, LCMT-1 and PME-1 signalling axis in adult rat ventricular myocytes

We initially aimed to determine the subcellular localisation of components comprising the LCMT-1/PME-1/PP2A signalling axis in ARVM. Previous studies have demonstrated that LCMT-1 protein expression in non-myocytes is restricted to the cytoplasm, whereas PME-1 has been shown to be predominantly expressed in the nucleus [35]. The subcellular fractionation of ARVM by digitonin/Triton X-100 based lysis is shown in Fig. 1 and confirms our own previous observation [23] that the PP2A_C subunit is predominantly localised to the cytoplasmic fraction of ARVM (Fig. 1A). Furthermore, Fig. 1A also shows that B55 α (PPP2R2A) regulatory subunit isoform that associates with the PP2A_{A/C} heterodimer in a methylation dependent manner [8] is similarly localised to the cytoplasmic fraction of ARVM. Importantly, both PME-1 (Fig. 1B) and LCMT-1 (Fig. 1C) protein expression appears to be almost exclusively localised in the cytoplasm of ARVM, with negligible localisation in the soluble membrane and insoluble fractions. This suggests that all components thought to regulate the carboxymethylation and methylation-dependent targeting of PP2A_C to cellular substrates are predominantly localised in the cytoplasm of ARVM.

G_iPCR-induced PP2A_C carboxymethylation in adult rat ventricular myocytes

Using an anti-methyl PP2A_C antibody (2A10) to detect the methylation status of the C-terminal leu309 of PP2A_C, data shown in Fig. 2A extends previously reported observations [22–24] that the stimulation of G_i-protein coupled A1.Rs by N6-cyclopentyladenosine (CPA) induces carboxymethylation of PP2A_C, by establishing the concentration-dependence of the response. In addition, we provide evidence shown that the Angiotensin II type 2 receptor (AT₂) agonist CGP42112 (Fig. 2B) and the muscarinic receptor agonist carbachol (Fig. 2C) also induce PP2A_C carboxymethylation in a concentration-dependent manner. Equal protein loading was determined by using a polyclonal anti-PP2A_C (FL-309) to detect both non-methylated and methylated forms of PP2A_C. These data suggest that PP2A_C carboxymethylation can be induced not only by the stimulation of A1.Rs but also by the stimulation of other G_iPCRs.

Using NaOH to remove the methyl moiety from the C-terminal leu309 of PP2A_C by saponification, we were able to demonstrate that the anti-methyl PP2A_C antibody (2A10) was no longer able to detect carboxymethylated PP2A_C. Methylation of leu309 within the TPDYFL C-terminal tail of PP2A_C masks the 4B7 antibody epitope and sterically interferes with antibody recognition of PP2A_C protein. We show in Fig. 3A that A1.R stimulation by CPA induces an increase in PP2A_C carboxymethylation, which is confirmed by two different antibodies raised against carboxymethylated PP2A_C (2A10) or demethylated PP2A_C (4B7). Having

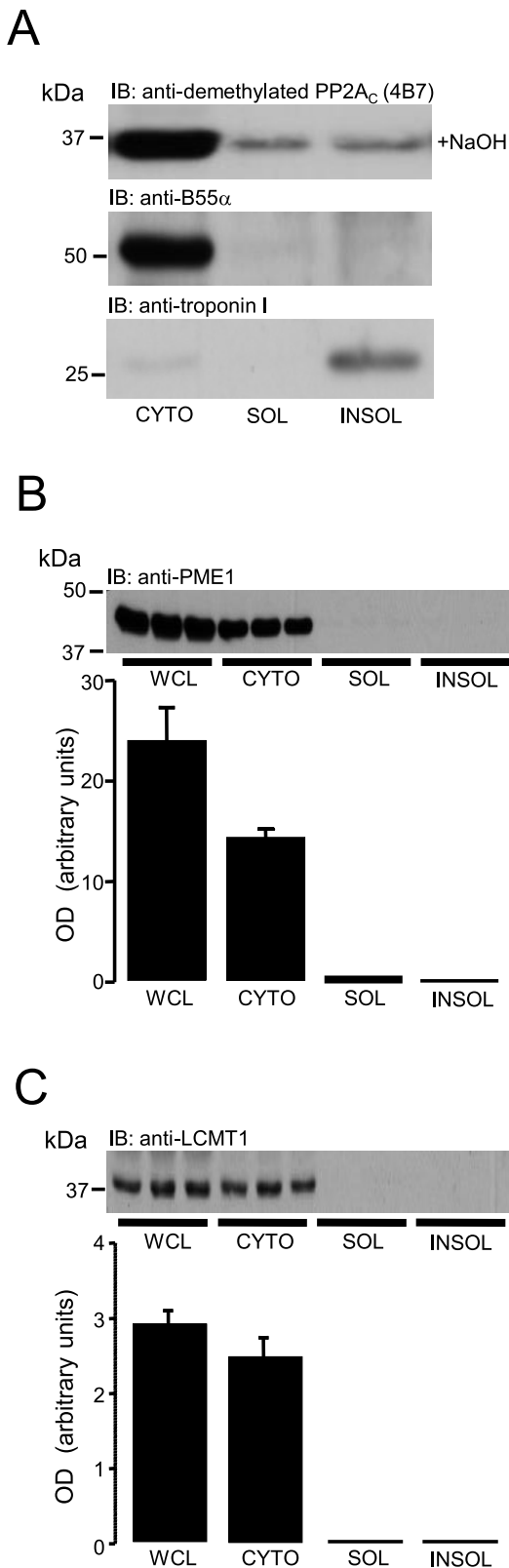


Figure 1. Subcellular localisation of proteins that regulate PP2AC carboxymethylation and targeting in ARVM. ARVM were initially lysed with a digitonin-based buffer to separate the cytoplasm and particulate fractions. The particulate fraction was then resuspended with a Triton X-100 based buffer to separate the soluble membrane and insoluble fractions by centrifugation. Samples were then analysed by

standard Western immunoblotting (IB). (A) Subcellular localisation of PP2AC and B55α protein in the cytoplasm (CYTO), soluble membrane (SOL) and insoluble (INSOL) fractions in ARVM. The presence of cardiac troponin I (cTnI) protein was to confirm the purity of the insoluble (INSOL) fraction. Subcellular localisation of PME-1 (B) and LCMT-1 (C) protein in the whole cell lysate (WCL), cytoplasm (CYTO), soluble membrane (SOL) and insoluble (INSOL) fractions in ARVM. All columns represent mean optical density (OD) values \pm SEM, $n=3$ individual experiments.

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used NaOH to remove the C-terminal (leu309) methyl moiety, the antibody raised against demethylated PP2AC (4B7) showed an equal amount of total PP2AC in all protein sample lanes. We believe that this constitutes an effective protocol for the determination of total PP2AC in any protein sample. Fig. 3B confirms that the increased level of PP2AC carboxymethylation in response to either CGP42112 or carbachol is also sensitive to saponification by NaOH.

Effects of CPA on cellular PP2AC binding partners

Several studies have shown that PP2AC can associate with several regulatory proteins such as PME-1 [14], LCMT-1 [16] and PKB [36] in non-myocytes. In support of this data, figure 4 shows that PP2AC does indeed exist in association with both PME-1 and LCMT-1 in unstimulated adult rat ventricular myocytes. Interestingly the data also demonstrate that although the exposure of ARVM to CPA does not alter the association between PP2AC and PME-1, it increases the association between PP2AC and LCMT-1. The data also suggest that, unlike in other cell types [36], there is no apparent association between cellular PP2AC and PKB in adult rat ventricular myocytes.

Role of Gβγ subunits in CPA-induced PP2AC translocation in adult rat ventricular myocytes

The classical effect of G_iPCR stimulation is to induce Gα_i-mediated inhibition of membrane bound adenylate cyclase [37], however, Gβγ subunits are known to mediate a range of intracellular signalling events of their own [38]. In this study we have exposed ARVM to increasing concentrations of CPA (0–100 μM) for 10 minutes and determined the translocation of PP2AC to the membrane-rich particulate fraction. Figure 5A shows that CPA significantly ($p<0.05$) increased the total PP2AC content within the particulate fraction of ARVM in a concentration dependent manner. To test the hypothesis that Gβγ subunits were mediators of the intracellular signalling cascade that led to G_iPCR-induced PP2AC translocation to the particulate fraction of ARVM in response to G_iPCR stimulation, we adenovirally expressed the Gα subunit of the ocular transducin GPCR, Gα_{t1} in these cells. Several studies have demonstrated that heterologous expression of the Gα_{t1} subunit in cells can sequester Gβγ subunits and switch off Gβγ subunit-dependent intracellular signalling [27,39,40]. Figure 5B illustrates the MOI-dependent levels of EGFP and Gα_{t1} protein co-expression and indicates that considerable heterologous expression of Gα_{t1} protein was achieved at an MOI of 50 pfu/ARVM. Hence, ARVM were infected with either a control EGFP-expressing or Gα_{t1}-expressing adenovirus (each at 50 MOI) and then exposed to 10 μM CPA for 10 minutes. Figure 5C shows that CPA-induced significant ($p<0.05$) PP2AC translocation to the particulate fraction in the presence of EGFP expression. However, this translocation was abrogated in ARVM infected to express Gα_{t1} in order to sequester Gβγ subunits. These data suggest that CPA-induced PP2AC translocation to the particulate fraction of ARVM is mediated by Gβγ subunits.

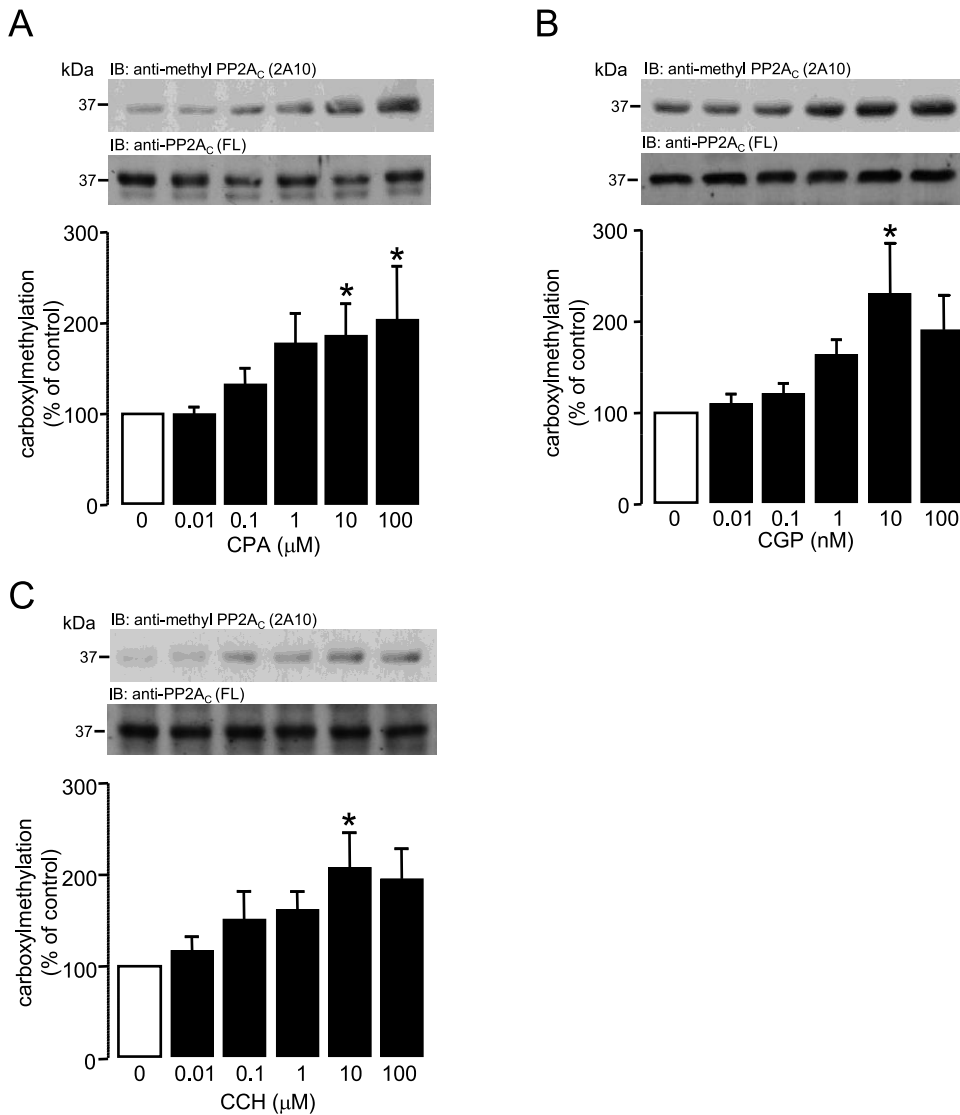


Figure 2. G_iPCR-induced carboxymethylation of PP2A_C in ARVM. ARVM were exposed to the G_iPCR agonists (A) CPA (0–100 μM), (B) CGP (0–100 nM) or (C) CCH (0–100 μM) for 10 minutes. Carboxymethylation of PP2A_C (as % of vehicle control) was detected by Western immunoblotting (IB) of the whole cell lysate with either an anti-methyl PP2A_C (2A10) or an anti-PP2A_C FL-309 (FL) antibody to determine equal protein loading. PP2A_C carboxymethylation was quantified by densitometry and all columns represent mean values ± SEM, n = 4–6 individual experiments, *p < 0.05 vs 0 (control group).

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CPA-induced activation of phosphoinositide 3-kinase (PI3K) and redistribution of PP2A_C in adult rat ventricular myocytes

Several studies have shown that G_iPCR stimulation can activate the PI3K family of lipid/protein kinases [41–43]. To determine that this occurs under our experimental conditions, we used PKB (ser473) phosphorylation status as a surrogate index of PI3K activity. CPA was found to induce significant (p < 0.05) phosphorylation of PKB at ser473 (Fig. 6A) and pretreatment of ARVM with the PI3K inhibitor LY294002 (20 μM) abrogated this response (Fig. 6B). These observations suggest that not only did the activation of G_i-coupled A1.Rs induce the phosphorylation of PKB (ser473), but that this occurred in a PI3K-dependent manner in ARVM. Importantly, Fig. 6C shows that pretreatment of ARVM with LY294002 (20 μM) abrogated CPA-induced PP2A_C translocation to the particulate fraction of ARVM, thereby

suggesting that G_iPCR-induced PP2A_C translocation is dependent on PI3K activation in ARVM.

Discussion

Several studies [22–24] have previously shown that the subcellular distribution of PP2A_C is predominantly restricted to the cytoplasm of ARVM, which we confirmed in the current study. Furthermore, the PP2A_C regulatory B subunit (PPP2R2A/B55α) which is known to associate exclusively with carboxyl-methylated PP2A_C [8] and form targetable PP2A heterotrimers, was also restricted to the cytoplasm of ARVM (Fig. 1A). This confirms that both PP2A_C and B55α are localised to the same cellular compartment of ARVM. The overall cellular carboxymethylation status of PP2A_C is thought to reflect the balance in activity of LCMT-1 and PME-1 towards their common substrate

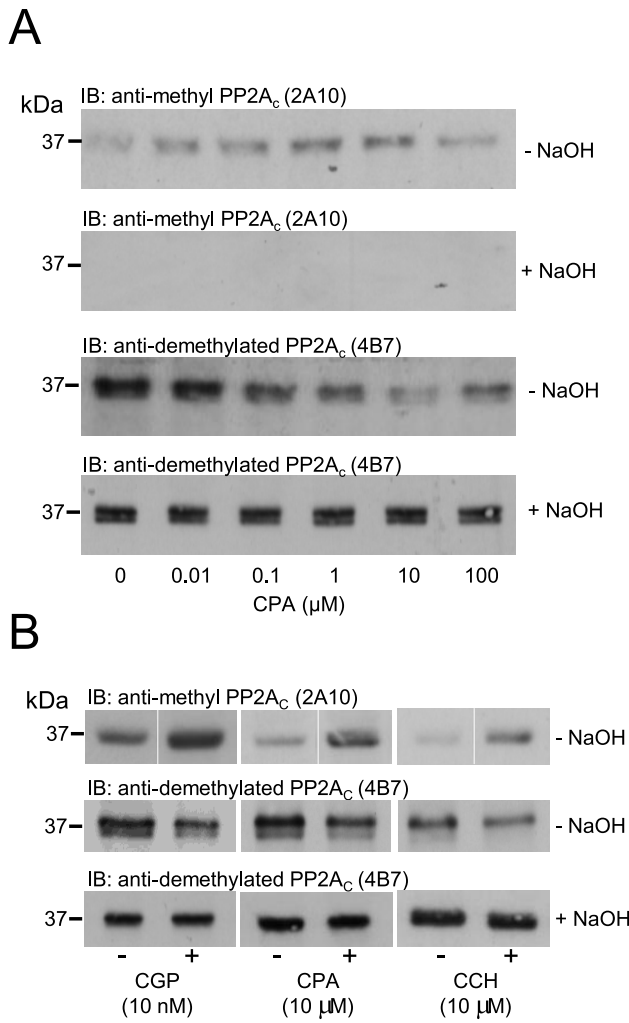


Figure 3. Saponification of carboxymethylated PP2AC by alkalinisation. ARVM were exposed to the A1.R agonist CPA (0–100 μM) for 10 minutes and PP2AC carboxymethylation was detected by Western immunoblotting (IB) with either an anti-methyl PP2AC (2A10) or demethylated PP2AC (4B7) antibody before (–NaOH) or after (+NaOH) saponification of the C-terminal leu309 methylation by 100 mM NaOH. (B) Carboxymethylation of PP2AC in response to CGP (10 nM), CPA (10 μM) or CCH (10 μM) as detected by Western immunoblotting with either an anti-methyl PP2AC (2A10) or demethylated PP2AC (4B7) antibody with or without treatment with 100 mM NaOH. Immunoblots are representative of 3 individual experiments. doi:10.1371/journal.pone.0086234.g003

PP2AC. X-ray crystallographic data suggest that the active sites of PP2AC, LCMT-1 [16] and PME-1 [14] can interact with each other to form tight PP2AC/LCMT-1 and PP2AC/PME-1 complexes, which regulate the methylation status of the C-terminal leu309 of PP2AC. These observations are supported by our own novel finding in ARVM, which suggest that cellular PP2AC does indeed basally form an association with both PME-1 and LCMT-1. Assuming that the activities of PME-1 and LCMT-1 remain constant, the increase in C-terminal leu309 PP2AC methylation as a result of G_i PCR stimulation, may be explained either by a reduced PME-1 or an increased LCMT-1 association with PP2AC. Our data suggests that the latter may be true as G_i PCR stimulation does not affect the association of PP2AC with PME-1 protein. This novel observation may explain the basis by which G_i PCR stimulation induces an increase in C-terminal

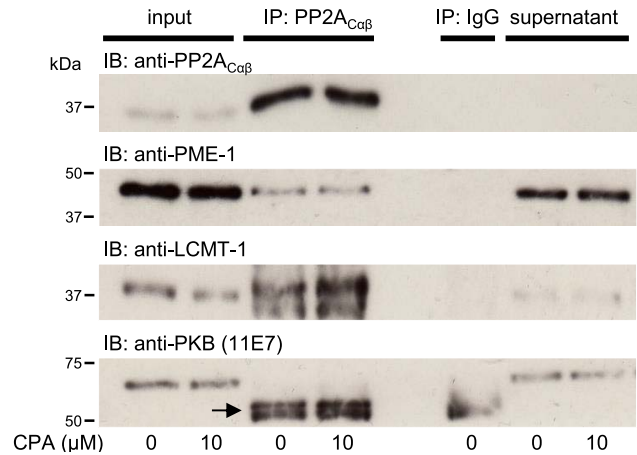


Figure 4. Effects of CPA on the association of PP2AC with cellular binding partners. ARVM were exposed to either vehicle (0.1% DMSO) control (0) or the A1.R agonist CPA (10 μM) for 10 minutes followed by lysis with an immunoprecipitation buffer. PP2AC $_{\alpha/\beta}$ was then immunoprecipitated from the resulting ARVM lysates and the immunocomplexes were then probed for the presence of PP2AC $_{\alpha/\beta}$, PME-1, LCMT-1 and PKB by standard Western immunoblotting. Immunoblots show the levels of the relevant proteins in the input, PP2AC $_{\alpha/\beta}$ immunocomplexes (IP: PP2AC $_{\alpha/\beta}$), non-specific immunocomplexes (IP: IgG) and supernatant (post-immunoprecipitation). The black arrow denotes detection of the immunoprecipitating IgG molecules. doi:10.1371/journal.pone.0086234.g004

leu309 carboxymethylation of PP2AC. Interestingly, immunoprecipitation of PP2AC removed virtually all PP2AC protein from the lysate (to undetectable levels) and a significant proportion of cellular LCMT-1 protein was immunoprecipitated along with it. Hence, the pool of cytoplasmic PP2AC in this complex may represent a reservoir of predominantly carboxymethylated PP2AC subunits. Although PME-1 protein was shown to associate with PP2AC, a significant proportion of PME-1 protein was still present in the immunocomplex supernatant (post-immunoprecipitation) and it is likely that the PP2AC in this complex is predominantly in the demethylated state [9,10].

As PP2AC subunits are the only known LCMT-1/PME-1 substrates, it is possible that cytoplasmic PP2AC restricts the localisation of LCMT-1, and to a lesser extent PME-1 protein, in the cytoplasm. PME-1 protein in human HeLa cells contains a functional 270KRKK273 nuclear localisation sequence (NLS), which explains why in this cell type PME-1 protein is thought to be predominantly localised in the nucleus together with demethylated PP2AC [35]. The human and rat PME-1 proteins are highly (98%) homologous, however, much of the variance that exists between these two PME-1 proteins, does so in the residues adjacent to the NLS. Rat PME-1 protein contains the sequence 268VNKRKK273 which differs to the human PME-1 protein sequence 268ISKRKK273. It is possible that the NLS contained within rat PME-1 protein is in some way dysfunctional due to the preceding valine/asparagine residues, which may partly explain why in ARVM, PME-1 protein is predominantly found in the cytoplasm and not the nucleus.

The carboxymethylation and subsequent translocation of PP2AC to the particulate fraction, in response to stimulated G_i protein coupled A1.Rs, within ARVMs was reported several years ago by the Hofmann laboratory [22,24,44]. In these studies, the phosphorylation of proteins in response to isoprenaline, present in the particulate fraction (troponin I and phospholamban), was found to be decreased by the stimulation of A1.Rs in a phosphatase-dependent manner. Our previous studies not only

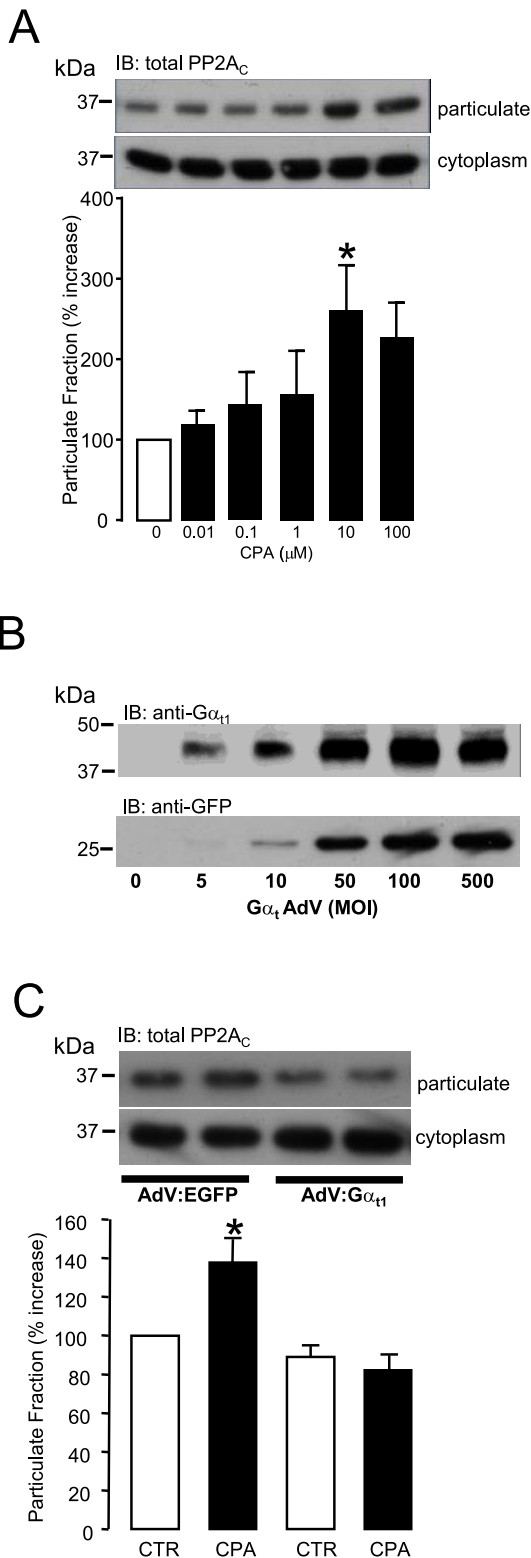


Figure 5. Role of Gβγ subunits in CPA-induced PP2A_C translocation. ARVM were lysed with a digitonin-based buffer to separate the cytoplasm and particulate fractions by centrifugation. Samples were then saponified with NaOH to abrogate any PP2A_C carboxymethylation. (A) Total PP2A_C content in the particulate fraction of ARVM in response to increasing concentrations of CPA (0–100 μM) was indexed by Western immunoblotting (IB) with an anti-demethylated PP2A_C antibody (4B7) following treatment with 100 mM NaOH.

Total PP2A_C content in the particulate fraction was quantified by densitometry. (B) Multiplicity of infection (MOI)-dependent co-expression of EGFP and Gα_{t1} protein in ARVM infected with the AdV:Gα_{t1}. (C) ARVM were infected with either the control AdV:EGFP or AdV:Gα_{t1} for 18 hours and then exposed to 10 μM CPA for 10 minutes. ARVM were then lysed and total PP2A_C content in the particulate fraction was indexed by Western immunoblotting (IB) with an anti-demethylated PP2A_C (4B7) antibody following treatment of samples with 100 mM NaOH. PP2A_C content in the particulate fraction was quantified by densitometry. All columns represent mean values ± SEM, n=4 individual experiments, *p<0.05 vs 0 (control group). doi:10.1371/journal.pone.0086234.g005

confirmed these observations, but identified an additional membrane bound protein (Na⁺/H⁺ exchanger isoform-1) as a novel PP2A_C substrate and demonstrated that the translocation of PP2A_C to the particulate fraction was sensitive to pertussis toxin [23]. Despite these studies suggesting that PP2A_C can be regulated by G_iPCR stimulation, very little has been reported since regarding the cellular mechanisms involved in the regulation of PP2A_C by inhibitory class GPCRs. A possibility that the PP2A_C translocation observed in ARVM may be A1.R-specific did exist. Hence, in this study we also chose to use other G_iPCR agonists such as carbachol and CGP42112 to target M₂.Rs and AT₂.Rs, respectively. Our data show that the stimulation of M₂.Rs and AT₂.Rs also induced PP2A_C carboxymethylation in a concentration-dependent manner. The data suggests that PP2A_C carboxymethylation is not a unique consequence of A1.R stimulation but can occur downstream of other G_iPCRs. To confirm that PP2A_C carboxymethylation in ARVM following exposure to CPA, carbachol or CGP42112 as detected by the anti-methyl PP2A_C antibody was genuine, we used NaOH to remove the methyl moiety by saponification from the C-terminal leu309 of PP2A_C [23,45,46]. Saponification abolished the signal detected by the anti-methyl PP2A_C antibody and equalised the signal in all lanes, as detected by the anti-demethylated (4B7) PP2A_C antibody. This confirmed that the methyl moiety on the C-terminal leu309 of PP2A_C conferred antibody epitope masking as previously reported [23,45,46].

These novel observations led us to next investigate mechanisms downstream of G_iPCR stimulation and we focused our attention on the role of Gβγ subunits in G_iPCR-induced cellular PP2A_C redistribution. We chose to sequester Gβγ subunits by adenovirally expressing the Gα_{t1} subunit of the ocular transducin GPCR, a protein not natively expressed in ARVM. Several studies [27,39,47] have successfully expressed Gα_{t1} subunits to implicate a role for Gβγ subunits in intracellular signalling events. Our data demonstrate that heterologous expression of Gα_{t1} subunits abrogated G_iPCR-induced PP2A_C translocation to the particulate fraction of ARVM. This is supported by studies suggesting the existence of a G_iPCR (A1.R)-Gβγ signalling hub in cardiac tissue. The stimulation of A1.Rs by chlorocyclopentyl adenosine in murine hearts was shown to activate phospholipase C in a Gβγ-dependent manner [48]. Furthermore, the stimulation of the dual (G_s/G_i) coupled β₂-adrenoceptor (β₂.AR) was reported to promote the survival of adult mouse cardiomyocytes following exposure to isoprenaline. In this study, the carboxyl terminus of β-adrenoceptor kinase (βARK-ct) a commonly used Gβγ inhibitor [49] abrogated isoprenaline-induced PKB activation and cellular survival, thereby implying that β₂.AR/G_i-induced cardiomyocyte survival involves Gβγ subunit activation [41]. Our novel data suggests that a proximal signalling step linking G_iPCRs to the regulation of PP2A_C cellular localization is mediated by Gβγ subunits.

Evidence [41,50] suggests that Gβγ subunits initiate intracellular signalling cascades via the activation of PI3K [38]. It has been

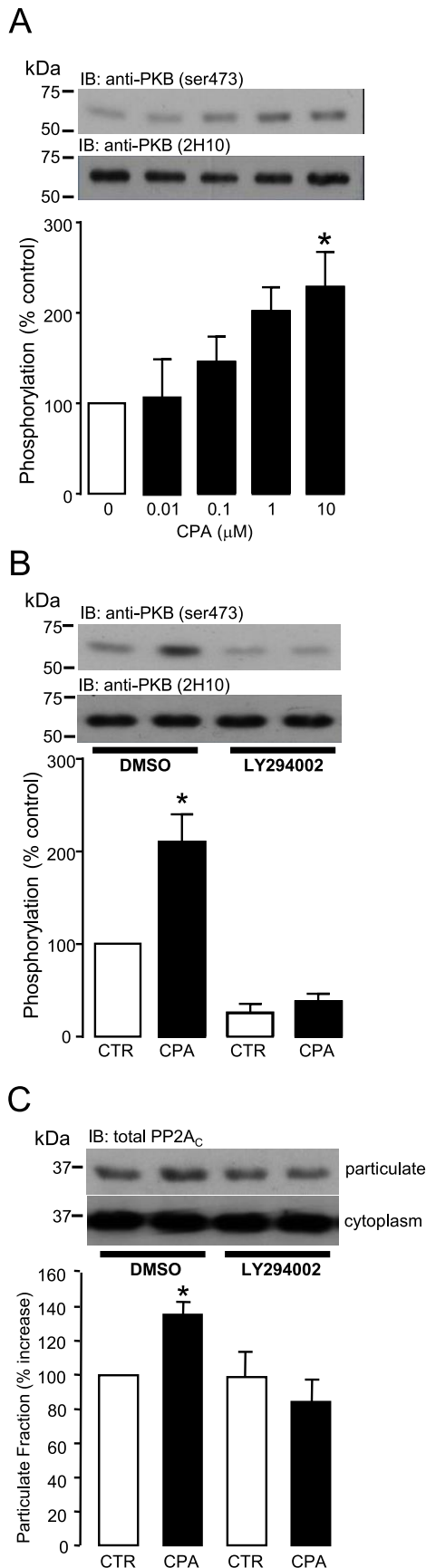


Figure 6. Role of PI3K in CPA-induced PP2AC translocation. (A) ARVM were exposed to the A1.R agonist CPA (0–100 μM) for 10 minutes and then lysed with SDS-PAGE Laemmli sample buffer for the determination of PKB phosphorylation (ser473) and total PKB by Western immunoblotting (IB). PKB (ser473) phosphorylation was quantified by densitometry. (B) ARVM were pretreated with either 0.1% DMSO (CTR) or 20 μM LY294002 for 30 minutes and then exposed to 0.1% DMSO (CTR) or 10 μM CPA for 10 minutes. PKB phosphorylation (ser473) and total PKB were then determined by Western immunoblotting (IB). PKB (ser473) phosphorylation was quantified by densitometry. (C) ARVM were pretreated with either 0.1% DMSO or 20 μM LY294002 for 30 minutes and then exposed to 0.1% DMSO (CTR) or 10 μM CPA for 10 minutes. ARVM were then lysed with 0.05% digitonin and fractionated by centrifugation. Samples were then saponified with NaOH to remove any PP2AC carboxymethylation. Total PP2AC content in the particulate fraction of ARVM was indexed by Western immunoblotting (IB) with anti-demethylated PP2AC (4B7) antibody following treatment with 100 mM NaOH. Total PP2AC content in the particulate fraction was quantified by densitometry. All columns represent mean values ± SEM, n=4 individual experiments, *p<0.05 vs 0 (control group).

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considered for some time that class IB PI3Ks can be activated by Gβγ dimers [51,52]. Class IB PI3Ks consist of a p110γ catalytic subunit associated with a regulatory subunit referred to as p101 [51]. The regulatory subunit p101 is central to the Gβγ-mediated activation of class IB PI3Ks [52]. Our observations with the PI3K inhibitor LY294002 suggest that A1.R stimulation induces PP2AC translocation to the particulate fraction via a Gβγ/PI3K signalling axis. In support of our observations, Zhu *et al* [41] reported that the G_i arm of the dual G_s/G_i-coupled β₂-adrenoceptor could also activate PI3K via Gβγ subunits in murine cardiomyocytes. Hence our data suggests that A1.Rs are linked to PP2AC by a Gβγ/PI3K signalling axis.

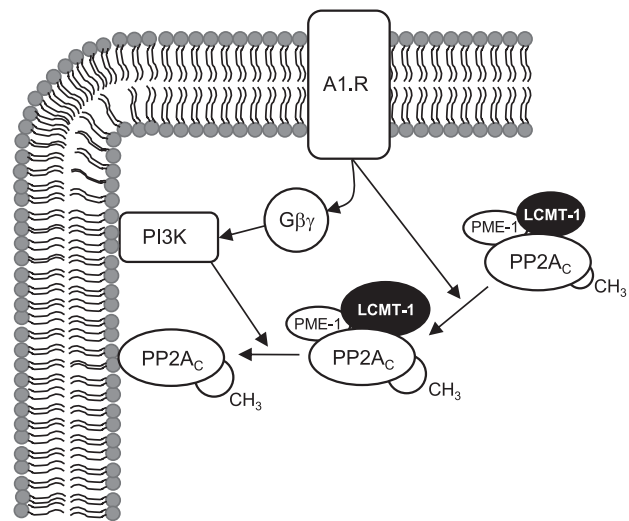


Figure 7. Suggested intracellular signalling mechanism (s) through which A1.Rs induce PP2AC translocation. Our data suggests that the stimulation of G_i protein-coupled adenosine A1 receptors by the agonist CPA increases the association of PP2AC with LCMT-1, thereby augmenting the leucine carboxymethylation status of PP2AC. The stimulation of G_i protein-coupled adenosine A1 receptors by the agonist CPA also elicits a cascade involving the release of Gβγ subunits which activate PI3K. Both of these intracellular signalling events coordinate and facilitate the association of PP2AC with the membrane-rich particulate compartment of ARVM.

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In conclusion, our data indicate that G_iPCR agonists may induce the carboxylmethylation of PP2A_C by increasing its association with the methyltransferase LCMT-1. G_iPCR agonists also mediate the disassociation of G α _i β γ heterotrimeric proteins and the released G β γ subunits in turn activate PI3K. The simultaneous activation of PI3K and induction of PP2A_C carboxylmethylation appear to coordinate the translocation of PP2A_C to the particulate fraction of ARVM in response to G_iPCR stimulation (see figure 7).

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Author Contributions

Conceived and designed the experiments: AKS. Performed the experiments: AKS AR. Analyzed the data: AR MA AKS. Contributed reagents/materials/analysis tools: MA AKS. Wrote the paper: MRL MA AR AKS.

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