Protein kinase A type I activates a CRE-element more efficiently than protein kinase A type II regardless of C subunit isoform

Øystein Stakkestad, Anja CV Larsen, Anne-Katrine Kvissel, Sissel Eikvar, Sigurd Ørstavik, Bjørn S Skålhegg

Abstract

Background: Protein kinase A type I (PKAI) and PKAII are expressed in most of the eukaryotic cells examined. PKA is a major receptor for cAMP and specificity is achieved partly through tissue-dependent expression and subcellular localization of subunits with different biochemical properties. In addition posttranslational modifications help fine tune PKA activity, distribution and interaction in the cell. In spite of this the functional significance of two forms of PKA in one cell has not been fully determined. Here we have tested the ability of PKAI and PKAII formed by expression of the regulatory (R) subunits RI\(\alpha\) or RII\(\alpha\) in conjunction with Ca1 or Cb2 to activate a co-transfected luciferase reporter gene, controlled by the cyclic AMP responsive element-binding protein (CREB) in vivo.

Results: We show that PKAI when expressed at equal levels as PKAII was significantly (p < 0.01) more efficient in inducing Cre-luciferase activity at saturating concentrations of cAMP. This result was obtained regardless of catalytic subunit identity.

Conclusion: We suggest that differential effects of PKAI and PKAII in inducing Cre-luciferase activity depend on R and not C subunit identity.

Background

Cyclic 3', 5'-adenosine monophosphate (cAMP) is a key intracellular signaling molecule, which main function is to activate the cAMP-dependent protein kinases (PKA) [1,2]. PKA is a heterotetrameric holoenzyme composed of two regulatory (R) and two catalytic (C) subunits, which is enzymatically inactive in the absence of cAMP. When two molecules of cAMP bind to each of the R subunits [3], the C subunits are released and activated to phosphorylate serine and threonine residues on specific intracellular target proteins [4,5]. Several PKA substrates have been identified of which the synthetic peptide Kemptide [6] and the naturally occurring substrate cAMP responsive element binding protein (CREB) are of the best characterized [7,8]. In primates, four genes encoding the R subunit and four genes encoding the C subunit, have been identified and designated RI\(\alpha\), RII\(\alpha\), RI\(\beta\), RII\(\beta\) and Ca, Cb, Cy and X-chromosome encoded protein kinase X (PrKX) [9].

Whereas no splice variants for RI\(\beta\) and RII\(\beta\) have been described, RII\(\alpha\) is transcribed from at least two different promoters. The first exons of the RII\(\alpha\) gene, exon 1a and 1b, give rise to alternatively spliced but identical proteins RII1a and RII1b [10]. RII1a and 1b mRNAs have been identified in most tissues and are differentially regulated by cAMP [11-13]. In the case of RII, it has been shown that RII\(\alpha\) in the human testis but no other tissues examined, is encoded with an alternative amino-terminal region [14]. No functional consequences of alternative splicing of RI and RII have been reported.

Several splice variants are transcribed from the Ca and the Cb genes (PRKCA and PRKCB) and include Ca1, Ca5, Cb1, Cb2, Cb3 and Cb4, in addition to an unknown number of abc forms of the Cb3 and Cb4 variants [15-20]. The major differences between the various C subunits are introduced through alternative use of exon 1 in the PRKCB and PRKCA genes, respectively [16,21,22]. In the case of Ca1 exon 1-1 encodes an
N-terminal stretch of 14 amino acids that have three sites that undergo co- and posttranslational modifications. At the very N-terminus a Gly is located that undergoes myristoylation in vivo [23]. C-terminal to the Gly an Asn is located that is partly deamidated in vivo leading to Ca1-Asp2 and Ca1-iso(β)Asp2 [24]. The third modification is PKA-autophosphorylation at Ser10 [25-27]. In the case of Cβ2, exon 1-2 encodes an N-terminal stretch of 62 amino acids that does not harbor sites for any of the modifications identified in Ca1. Instead, the Cβ2 N-terminus contains a stretch of hydrophobic amino acids that form an amphiphatic α-helix displayed as a hydrophobic surface [20]. Ca1 and Cβ1 are more than 90% identical at the amino acid level and are ubiquitously expressed. CaS has only been identified in sperm cells [28], Cβ2 is predominantly expressed in lymphoid cells [29,30], and Cβ3 and Cβ4 and their abc variants are mainly expressed in neuronal tissues [15,16].

It is assumed that any known C subunit may associate with RI and RII to form PKAI and PKAII, respectively [9]. This has raised the question of the biological significance of PKAI and II holoenzymes containing various C isoforms within the same cell. Whereas no reports have been published on the functional consequences of holoenzymes formed with various C subunits, it has been demonstrated that several cell types expressing RII variants results in more discrete defects, affecting differentiation of adipose tissue and neural functions [35-37]. The levels of RI and RII as well as tissue- and subcellular expression varies. They also show differential affinities for A-kinase anchoring proteins (AKAP).

Furthermore, when determining the structure of the PKA holoenzymes it was found that RI and RII contact the substrate binding site of the C subunit either as a true PKA substrate (RII) or as a pseudosubstrate (RI) due to autophosphorylation of RII but not RI at Ser95 [38,39]. Despite these differences an explanation for biological differences at the cellular level between RI and RII are not fully appreciated [40,41]. However, it should be noted that RII autophosphorylation modulates AKAP-RII interaction in cardiac cells, leading to altered down-stream substrate phosphorylation and Ca2+ dynamics [42].

To investigate biological differences between RI and RII and to demonstrate if such differences are dependent on C subunit identity we formed PKAI and PKAII by co-transfecting 293T cells with either RIIα or RIIα together with Ca1 and Cβ2, respectively. This demonstrated that PKAI was superior to PKAII in activating a cAMP responsive element regardless of whether the holoenzyme contained Ca1 or Cβ2. Our results contribute to understand the functional significance of two PKA holoenzymes but not various C subunits expressed in the same cell.

**Results**

To test for differential roles of PKAI and PKAII expressed in one cell we tested if markedly different C subunits released from RI and RII are equally effective in regulating in vivo substrate phosphorylation. We chose the cell line 293T as a model system since they express RIIα and RIIα associated with Ca1 (Figure 1A, left panel), and not RIIβ and RIIβ (Figure 1A, right panel). In these cells PKAI and PKAII are distinctly located to the cytosol and Golgi-centrosomal area, respectively as demonstrated by immunostaining using anti-RIIα (red) or anti-RIIα (green) (Figure 1B). Co-immunostaining with anti-C demonstrated that Ca1 localization corresponded to RII subunit localization. We also observed a weak nuclear staining of the C subunit in the absence of cAMP (Figure 1C), whereas in the presence of the cAMP analogue, 8-CPT-cAMP (340 µM) an increase in nuclear staining was observed (Figure 1C). We concluded that the 293T cells represented a suitable model system to study isoform differences between PKAI and PKAII formed with different C subunits.

To obtain 293T cells dominated by either PKAI or PKAII expression, we formed holoenzymes by transient transfection of plasmids over-expressing either RIIα or RIIα (pDeRIIα or pExRIIα) in combination with either Ca1 or Cβ2 (pDeCa1 or pDeCβ2). For some experiments the cells were also transfected with a vector expressing Luciferase controlled by a cAMP responsive element. C subunit activity was tested in vitro using Kemptide as a substrate [43,44]; and in vivo using the Cre-Luciferase reporter system [45]. This revealed a dose-dependent increase in PKA-specific catalytic activity against Kemptide for both pDeCa1 and pDeCβ2 with a maximum at 5600 ng DNA (Figure 2A). The luciferase response was bell shaped and reached a maximum for pDeCa1 and pDeCβ2 at 1400 and 2800 ng DNA, respectively (Figure 2B). Next, we titrated the plasmids expressing RI and RII by transfecting 0-1280 ng of the plasmids pDeRIIα and pExRIIα, respectively (Figure 2C, D).

Twenty four hours after transfection cells were lysed and R subunit levels were measured by immunoblotting and [3H]-cAMP-binding. This revealed an increase in a 49 kDa immunoreactive band as well as increased [3H]-cAMP-binding that coincided with the amount of plasmid transfected (pDeRIIα, Figure 2C). The same was the case when transfecting pExRIIα (Figure 2D). Together this demonstrated a dose-dependent expression of both RIIα and RIIα.
Based on these transfections and earlier experiments (results not shown), we next formed PKA holoenzymes by R and C co-transfections. We aimed at transfecting R plasmids to levels where C activity in the absence of cAMP were at basal levels, implying levels of R able to associate with all C subunits. 293T cells were co-transfected with a fixed amount of either pDeCa1 (300 ng) or pDeCβ2 (1400 ng) together with increasing amounts of pDeRα (0-1280 ng, Figure 3A, B) and pExR1β (0-1280 ng, Figure 3C, D), respectively. Cell extracts were adjusted to 1 mg total protein/mL and total C subunit activity measured in the presence and absence of 8-CPT-cAMP.

**Figure 1** PKAI (RαCa1) and PKAII (RβCa1) are expressed in 293T cells. (A) Cell extracts of 293T cells (40 μg protein/lane) were analyzed by immunoblotting using a pan-anti-C antibody (upper left panel) and anti-Rα and anti-Rβ (lower left panels). The levels and identities of 293T cell C and R subunit expression were compared to human peripheral blood lymphocytes (hPBL) revealing expression of Ca1, Rα and Rβ. No detectable levels of Rβ and Rβ were identified when compared to extracts of human temporal cortex (hTempCortex, right panels). (B) Immunofluorescence analysis of PKA Rα and Rβ in 293T cells. Rα (Anti-Rα, red) is expressed diffusely in the cytosol and Rβ (Anti-Rβ, green) is expressed in the Golgi-centrosomal area of 293T cells. (C) Immunofluorescence analysis of PKA C subunits in 293T treated without (-) or with (+) 340 μM 8-CPT-cAMP.
absence of 7.14 μM cAMP. This demonstrated that Cα1-specific kinase activity was inhibited down to basal levels in the absence of cAMP at 640 ng pDeRI (Figure 3A), which was equal to 28 ± 1.4 pmol RIIα/mg total protein (Table 1). In the case of Cβ2-specific activity it was down to basal levels in the absence of cAMP at 80 ng pDeRIα (Figure 3B) which was equal to 11.8 ± 2.7 pmol RIIα/mg total protein (Table 1). For RIIα, 320 ng pExRIIα was required for optimal Cα1 inhibition (Figure 3C), which was equal to 16.2 ± 0.5 pmol RIIα/mg total protein.
(Table 1). Finally, 80 ng pExRIIα was required to inhibit Cβ2 activity to basal levels (Figure 3D) which was equal to 9.6 ± 2 pmol RIIα/mg total protein. In order to compare in vitro and in vivo PKA activity, protein extracts were analyzed against Kemptide phosphorylation and luciferase activity after transfection with Cage-Cre-Luciferase (700 ng) together with either 300 ng pDeCα or 1400 ng pDeCβ and increasing amounts of pDeRIα and pExRIIα (160-1280 ng DNA, Figure 4A-D). In these experiments psv-β-Galactosidase (1000 ng) was used for normalization (see Methods). This showed that luciferase activity induced by Cα1 and Cβ2 was completely inhibited by both RIα and RIIα at doses above or equal to 640 ng plasmid DNA.

The experiments in Figures 3 and 4 depict that Cβ2 activity is fully inhibited at lower amounts of R than Cα1 is. This may imply that Cα1 is enzymatically more active than Cβ2 or simply that Cβ2 is more unstable than Cα1 in the absence of R. A previous report shows that the C subunit in its free active form is more rapidly degraded than C complexed with the R subunit dimer [46]. To test if Cα1 and Cβ2 display differential stability, identical amounts of Cα1 and Cβ2 plasmids were transfected alone or with 1280 ng of pDeRα. This confirmed (Figure 5 bars 2 and 3) that in the absence of Rα total Cβ2 activity is significantly (* p< 0.05) lower compared to Cα1. This was not the case when Rα was co-transfected with the two C subunits. In this case both Cα1 and Cβ2 activities were
increased, however, to comparable levels after stimulation with cAMP (bars 5 and 7, ns). This demonstrated that RIα has a stabilizing effect on both C subunits. However the effect was more pronounced for Cb2 than Ca1 indicating that Cb2 is more unstable than Ca1 in the absence of R.

The results from Figures 3 and 4 demonstrated that we had obtained cell systems dominated by either PKAI or PKAII. Hence, the effects of PKAI and PKAII on in vitro (Kemptide) and in vivo (CREB) phosphorylation could be tested. For these experiments we used amounts of RIα and RIIα required for complete inhibition of Ca1 and Cb2 respectively.

After 24 hours cell extracts were diluted to 1 mg total protein/mL and analyzed for cAMP dose-dependent induction of PKA kinase activity against Kemptide (Figure 6A, C). Both RIα and RIIα were able to inhibit Ca1 and Cb2 kinase activity completely in the absence of cAMP. When increasing the concentrations of cAMP from 5 to 5000 nM, kinase activity was peaking, in the case of Ca1 at 100 nM cAMP when co-expressed with RIα and between 500 and 5000 nM when co-expressed with RIIα. In the case of Cb2, maximum activity was achieved at concentrations between 500 and 5000 nM cAMP when co-expressed with both RIα and RIIα. We further analyzed C subunit activity in vivo by measuring luciferase activity. Activity was measured after stimulation of the transfected cells with increasing concentrations of 8-CPT-cAMP (0 - 320 μM) for 1 hour prior to harvesting. We observed that activity associated with Ca1 and Cb2 released from both RIα and RIIα increased in a dose-dependent manner, reaching maximum between 160 and 320 μM 8-CPT-cAMP (Figure 6B, D). However, a more than two fold higher activity was observed against CREB when Ca1 and Cb2 were released from RIα than from RIIα. Together these results indicated that the ability of C to phosphorylate nuclear substrates in vivo at saturating concentrations of cAMP when associated with PKAII was lower than...
when associated with PKAI. This was apparent despite that total C subunit activity in vitro was comparable and protein concentrations were equal (Figure 6A to 6D). Since these results were seen regardless of C subunit isoform we suspected that the differences observed were associated with R subunit identity. To quantify the different efficacy of PKAI and PKAII to phosphorylate CREB in vivo, we therefore co-transfected pDeRI (640 ng) and pExRII (320 ng) with Cα1 (300 ng pDeCα1) and monitored [3H]-cAMP binding. This showed equal activities (Figure 7A) and hence comparable levels revealed as 22 ± 1.5 and 23 ± 1.5 pmol per mg total protein of RIα and RIIα, respectively. We next determined C subunit activity in vitro after transfecting cells as described in Figure 7A, and in the absence (0 nM) and presence of two concentrations of cAMP (5 and 5000 nM). This revealed basal activity in the absence, and low level activity in the presence of 5 nM cAMP whereas 5000 nM cAMP resulted in comparable high levels of total C subunit activity released from both PKAI and PKAII (Figure 7B). The C activities were equal to 25 ± 1.4 and 24.2 ± 2.9 pmol Cα1 per mg total protein for PKAI and PKAII, respectively (Table 2). This concluded that PKAI and PKAII were expressed at comparable levels under the present conditions. The latter was substantiated by a calculated R to C ratio close to 1 for both RIα versus Cα1 (ratio 0.88) and RIIα versus Cα1 (ratio 0.96, Table 2).

In lymphoid cells, it has been demonstrated that R subunits are more stable in the holoenzyme form compared to the free R subunit [47]. To test if the presence of Cα1 alone and in conjunction with cAMP would influence R subunit levels we transfected 293T cells with either pDeRIα (640 ng) or pDeCβ2 (1400 ng) in conjunction with 1280 ng (bars 4 to 7) or without (bars 1-3) pDeRα for 24 h. Cell extracts were adjusted to 1 mg total protein/mL and assayed for PKA-specific phosphotransferase activity in the absence (- cAMP) and presence (+ cAMP) of 320 nM cAMP. Data points represent pmol ATP transferred/min/mg protein) +/- SD (n = 3). The relative activities of Cβ2 and Cα1 in the absence of Rα were significantly different (*p < 0.02, bars 2 and 3) When Cα1 and Cβ2 were co-transfected with Rα the relative activities were indistinguishable (bars 5 and 7, ns).
stimulation appeared not to influence R subunit levels and thus the cAMP sensitivity of the system.

Based on our observations (Figure 6B and 6D), we transfected cells as described in Figure 7B with equal amounts of PKAI and PKAII and monitored luciferase activity after stimulation with two concentrations of 8-CPT-cAMP (1 and 320 μM) for 1 hour before harvesting. As depicted in Figure 7D 320 μM 8-CPT-cAMP induced more than a 13-fold increase in luciferase activity when associated with RIα compared to untreated cells. When associated with RIIα the induction was 3-fold. This difference was reflected in a relative induction of luciferase activity which was nearly twice as high for PKAI compared to PKAII (1.94 fold, p < 0.01).

### Discussion

Despite that PKAI and PKAII are located to different areas when expressed in the same cell, it is believed that when dissociated by cAMP, the C subunits are all released to phosphorylate relevant substrates both in the cytosol and nucleus [48]. We formed PKAI and PKAII holoenzymes by co-transfecting 293T cells with Rlα or RIIα together with either Ca1 or Cβ2.

We found that C subunits, irrespective of isoform, appeared more efficient in inducing Cre-luciferase when released from PKAI than PKAII.

To monitor total PKA activity in vitro and in vivo we applied cAMP and the cAMP analogue 8-CPT-cAMP. In vitro activation of PKA by cAMP was done to
monitor if we had achieved comparable amounts of PKAI and PKAII in our experiments. For monitoring in vivo endogenous activity 8-CPT-cAMP was used because it has cell membrane permeable properties and is resistant to phosphodiesterase degradation [49]. The observation that cells transfected with PKAI induced higher levels of luciferase activity upon 8-CPT-cAMP stimulation than cells transfected with PKAII may have been due to relative affinities of the cAMP analogue. We consider this unlikely since 8-CPT-cAMP is a B-site selective cAMP analogue with higher affinity for RII than RI [49]. Further support for 8-CPT-cAMP as a competent activator of PKAII in vivo is found in that the concentration of 8-CPT-cAMP used is capable of displacing the C subunit from the RII subunit interacting with the centrosome in vivo in U2OS cells [50]. Taken together we conclude that 8-CPT-cAMP is fully capable to activate PKAII and does not selectively activate PKAI, implying that PKAII is less potent compared with PKAI in inducing Cre-luciferase activity.

An explanation for the biological significance of the phenomenon observed may rely on several factors. Despite that 25% of PKA is undissociated even in the presence of saturating concentrations of cAMP [51] it may not account for the differences we observed since this is observed for both PKAI and PKAII. However, it

| PKA subunits | [R] | [C] | R/C ratio |
|--------------|-----|-----|---------|
| Holoenzyme   |     |     |         |
| PKAI         | 22 ± 1.5 | 25 ± 1.4 | 0.88   |
| PKAII        | 23 ± 1.5 | 24 ± 2.9 | 0.96   |

*Concentration of R and C subunit (pmol/mg protein) were determined at saturating concentrations of cAMP and assuming two cAMP binding sites per R subunit and 600 pmol phosphate transferred by pure bovine C per min per mg [63].
has been demonstrated that cAMP-dissociated RII and C reassociate much faster and to a much greater extent than RI and C. In fact it has been suggested that C does not really leave RII under physiological conditions due to a rapid reassociation [52]. Hence, incomplete dissociation of C subunit from RII even at saturating concentrations of cAMP could be a mechanism explaining the phenomena observed here. Moreover, the biological significance of differential effects of activating PKAI and PKAII independent of C subunit identity may be multiple. Recently a paper by Di and co-workers [53] demonstrated that PKAI and PKAII define distinct intracellular signaling compartments. They demonstrated that PKAI and PKAII activity were regulated by distinct, spatially restricted cAMP signals generated in response to specific G protein-coupled receptors and which were regulated by unique subsets of the cAMP degrading phosphodiesterases.

We observed that Ca1 was more active than Cβ2 when expressed in non-holoenzyme form. This may suggest differential Kd of Ca1 and Cβ2 against RI and RII. This suggestion was supported in that the amount of R plasmid required for complete inhibition of Ca1 and Cβ2, respectively, was higher for RI compared to RII regardless of C subunit identity (28 pmol R1α/mg protein and 15 pmol R1α/mg protein for Ca1 versus ~12 pmol R1α/mg protein and ~10 pmol R1α/mg protein for Cβ2). However, we also observed that Cβ2, but not Ca1 activity was stabilized when co-transfecting with the R subunit implying that the differences observed is due to protein instability of the Cβ2 subunit and not lower Kd for the R subunit.

The latter is supported by the observation that R and C dissociation by cAMP in vivo promotes degradation of C subunits through posttranslational mechanisms which may involve proteasome action [54]. Furthermore, it has been shown that Ca1 and Cβ1 have identical Kd values for RI [55]. To what extent Cβ2 is more sensitive to proteasome degradation than Ca1 is not known. It should however be noted that the marked differences between the Ca1 and Cβ2 at the N-terminus has been implicated in C subunit stability. For Ca1 it has been demonstrated that the α-helix and Trp 30 are vital moieties for Ca1 stability. This correlates with the location of the N-terminal at the cleft interface where it orients the C-helix in the small lobe and the activation loop in the large lobe so that these subdomains are aligned in a way that allows for correct configuration of residues at the active site [56]. Moreover, we did not demonstrate a relative difference in potency of Cβ2 versus Ca1 in inducing Cre-luciferase activity irrespective of association with R1α or R1α. The latter may suggest that Ca1 and Cβ2 behave identically in regulating Cre-luciferase activities. Hence, we concluded that the differential effects of PKAI and PKAII on luciferase activity detected in the present work are associated with R subunit but not C subunit. The latter was unexpected since it has been speculated if the marked sequence differences at the N-terminus will influence PKA holoenzyme features such as localization. The latter has previously been demonstrated in that the N-terminus of Ca1 is implicated in subcellular anchoring to A-kinase interacting protein 1 (AKIP1) [57]. Furthermore, at the N-terminal end the myristoyl moiety, which binds to a hydrophobic pocket on the surface of the large lobe when Ca1 subunit is in the holoenzyme form [58,59], is exposed to the surroundings upon binding to RII. This makes the holoenzyme more hydrophobic [60]. In addition, whereas the N-terminal Asn moiety, is involved in fine-tuning of the enzyme distribution within the cell in vivo [61], Ser10 phosphorylation is known to introduce electro statically mediated forces which may help C to remain soluble even when myristoylated [62-64]. Together this implies the N-terminal of Ca1 to contribute to regulation and tuning of subcellular targeting. Despite lack of experimental evidence the N-terminal amphipathic α-helix in Cβ2 has been proposed to function as a targeting domain for Cβ2 in vivo [20]. Despite the obvious differences between Ca1 and Cβ2 we did not observe any experimental evidence on the C subunits contributing to understand the differential effects of PKAI and PKAII.

In perspective, the various reports referred to here [51-53] together with our observations demonstrate differential activities and regulation by PKAI and PKAII which may add to understand the biological significance of two PKA holoenzymes expressed in one cell.

**Conclusions**

This study is important because it points to how tissue-dependent expression of genes encoding subunits of PKA achieve specificity in the cAMP signaling pathway. Our work shows that transfected PKAI holoenzymes are more efficient than PKAII in phosphorylating CRE elements in vivo regardless of C subunit identity. Furthermore we show that Cβ2 appear more stable in the presence of R subunit than Ca1.

**Methods**

**Cell culture**

293T HEK cells were maintained in RPMI medium 1640 (Sigma) containing 10% (v/v) Fetal Bovine Serum (Sigma), 2 mM L-Glutamine (Sigma) 1% Non-essential amino acids (Gibco), 1% Na-Pyruvat (Gibco) and 1% (v/v) Penicillin/Streptomycin (Sigma). The cells were subcultured three times weekly. Twenty hours before transfection 293T cells were grown in 6 well plates from a population of 0.7 × 10⁶ cells per well containing 1.5 mL RPMI medium without Penicillin/Streptomycin. Plates were kept at 37°C in a humidified atmosphere under 5% CO₂.
Generation and expression of PKA vectors

pEF-DEST 51™ (Invitrogen) expression vectors encoding human regulatory and catalytic subunits Rα, Ca1 and Cβ2 were created using Gateway LR Clonase Reaction® (Invitrogen) and transformed into Library™ efficiency DH5α™ Competent cells (Invitrogen). Plasmid pBlue-script containing RIIα encoding fragment was digested with Eag I (New England Biolabs), ligated using T4 Ligase (Promega) in plasmid pExchange 6A (Promega) previously digested with Not I (Promega), and transformed into Ultramax® DH5α™ Competent cells (Invitrogen). Plasmids expressing catalytic subunits Ca1 or Cβ2 or/and regulatory subunits RIIα or Rα herby termed pDeCa1, pDeCβ2, pDeRα, and pExRIIα where transfected using Lipojectamine 2000 (Invitrogen). In order to facilitate a reporter system, plasmids expressing Luciferase reporter gene and β-Galactosidase as a normalization control was co-transfected with R subunit and/or C subunit in constant amounts (0.7 μg Cage-Cre-Luciferase reporter vector and 1 μg Psv-β-Galactosidase vector) in all wells except wells kept as “mock” controls. A vector without insert was used to keep the amount of plasmid DNA transfected constant. Cells were stimulated with 8-CPT-cAMP for 1 or 4 hours (specified in the text) before being harvested 24 hours post transfection.

Immunoblot analysis

Immunoblotting was performed as previously described [15]. Membranes were incubated with mouse monoclonal anti-RIIB (cat # 610625, BD Transduction laboratories) at 1:250 dilution, polyclonal rabbit anti-RIIα (cat # SC-907, Santa Cruz Biotechnology, Inc.), anti-RIIα (cat # 612243, BD Transduction laboratories) at 1:400 dilution or mouse monoclonal anti-Rα (Clone 4D7, [65]) at 1:300 dilution. Immunoreactive proteins were detected with HRP-conjugated secondary antibodies (ICN Diagnostics) and SuperSignal® West Pico Chemiluminescent (Pierce).

Phosphotransferase assays

PKA-specific catalytic activity was determined as described previously [66]. Molar amounts of C subunit were determined assuming 600 pmol phosphate transferred per min per mg pure bovine C.

Luciferase assay

Briefly, 24 hours post transfection cells were harvested, lysed by sonication, and samples adjusted to equal protein concentrations (1 mg/mL). Lysates were added appropriate buffer containing 270 μM Coenzyme A (Boehringer), 530 μM ATP (Boehringer), 470 μM Luciferin (SynChem), and immediately placed in a Luminometer (TD20/20, Turner Designs). Luminosity was measured after 2 seconds delay at 560 nm for 15 seconds with 20.1% of intensity. Samples in the high end of luminosity were used to create a standard curve to ensure measurement in the linear range.

R-binding assay

The level of R-subunits was determined by specific [3H]-cAMP binding in homogenates from transfected 293T cells as previously described [15]. Molar amounts of R subunits were calculated assuming two cAMP binding sites per R subunit.

Indirect Immunofluorescence (IF)

IF of 293T cells were performed as previously described [67]. Antibodies against R I (Clone 4D7, [65]) and RII (cat # 612243, BD Transduction laboratories) were diluted (see figure legend). The anti-C antibodies were rabbit polyclonal anti-Cα 1:100 (cat # sc 903, Santa Cruz Biotechnology, Santa Cruz, CA).

Statistics

Data are presented as means ± s.e.m and were analyzed by unpaired two-tailed t test or by one-way analysis. A value of <0.05 was considered statistically significant. All statistics were calculated by the Graphpad prism 5.02 program.

List of abbreviations

C: catalytic subunit; CREB: cAMP-responsive element binding protein; PKA: protein kinase A; R: regulatory subunit of PKA.

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Author details

1. Department of Nutrition, Institute for Basic Medical Sciences, University of Oslo, Sognsvannsveien 9, P.O. Box 1112 Blindern, N-0317 OSLO, Norway.
2. Department of Biochemistry, Institute for Basic Medical Sciences, University of Oslo, Sognsvannsveien 9, P.O. Box 1112 Blindern, N-0317 OSLO, Norway.
3. Department of Pathology, The Norwegian Radium Hospital, Oslo University Hospital, Ullevål Avenue 70, N-0310 Oslo, Norway.
4. Department of Oncology, Ullevål Hospital, Oslo University Hospital, Kirkeveien 166, P.O. Box 4950, Nydalen N-0424, OSLO, Norway.

Authors’ contributions

ØS carried out most of the experiments, participated in the design of the study and in drafting the manuscript and preparing it for submission. ACVL participated in the experiments, provided technical assistance and contributed in criticizing the manuscript. AK performed indirect immunofluorescence experiments and contributed in criticizing the manuscript. SE participated in indirect immunofluorescence experiments and provided technical assistance. SJ conceived the design of the study, helped in its coordination and contributed in criticizing the manuscript. BSS conceived the design of the study, helped in its coordination and wrote the manuscript. All authors read and approved the final manuscript.

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