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

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## 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 C $\alpha$ 1 or C $\beta$ 2 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 $\alpha$ ,

RI $\beta$ , RII $\alpha$ , RII $\beta$  and C $\alpha$ , C $\beta$ , C $\gamma$  and X-chromosome encoded protein kinase X (PrKX) [9].

Whereas no splice variants for RI $\beta$  and RII $\beta$  have been described, RI $\alpha$  is transcribed from at least two different promoters. The first exons of the RI $\alpha$  gene, exon 1a and 1b, give rise to alternatively spliced but identical proteins RI $\alpha$ 1a and RI $\alpha$ 1b [10]. RI $\alpha$  1a 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 C $\alpha$  and the C $\beta$  genes (PRKCA and PRKCB) and include C $\alpha$ 1, C $\alpha$ S, C $\beta$ 1, C $\beta$ 2, C $\beta$ 3 and C $\beta$ 4, in addition to an unknown number of abc forms of the C $\beta$ 3 and C $\beta$ 4 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 C $\alpha$ 1 exon 1-1 encodes an

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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  $\text{C}\alpha 1\text{-Asp2}$  and  $\text{C}\alpha 1\text{-iso}(\beta)\text{Asp2}$  [24]. The third modification is PKA-autophosphorylation at Ser10 [25-27]. In the case of  $\text{C}\beta 2$ , exon 1-2 encodes an N-terminal stretch of 62 amino acids that does not harbor sites for any of the modifications identified in  $\text{C}\alpha 1$ . Instead, the  $\text{C}\beta 2$  N-terminus contains a stretch of hydrophobic amino acids that form an amphiphatic  $\alpha$ -helix displayed as a hydrophobic surface [20].  $\text{C}\alpha 1$  and  $\text{C}\beta 1$  are more than 90% identical at the amino acid level and are ubiquitously expressed.  $\text{C}\alpha 5$  has only been identified in sperm cells [28],  $\text{C}\beta 2$  is predominantly expressed in lymphoid cells [29,30], and  $\text{C}\beta 3$  and  $\text{C}\beta 4$  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  $\text{RI}\alpha$  are highly proliferative and may also be associated with malignancy [31-34]. Using a genetic approach it has also been demonstrated that constitutive ablation of  $\text{RI}\alpha$  but not  $\text{RI}\beta$  is prenatal lethal whereas ablation of the 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  $\text{Ca}^{2+}$  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  $\text{RI}\alpha$  or  $\text{RII}\alpha$  together with  $\text{C}\alpha 1$  and  $\text{C}\beta 2$ , respectively. This demonstrated that PKAI was superior to PKAII in activating a

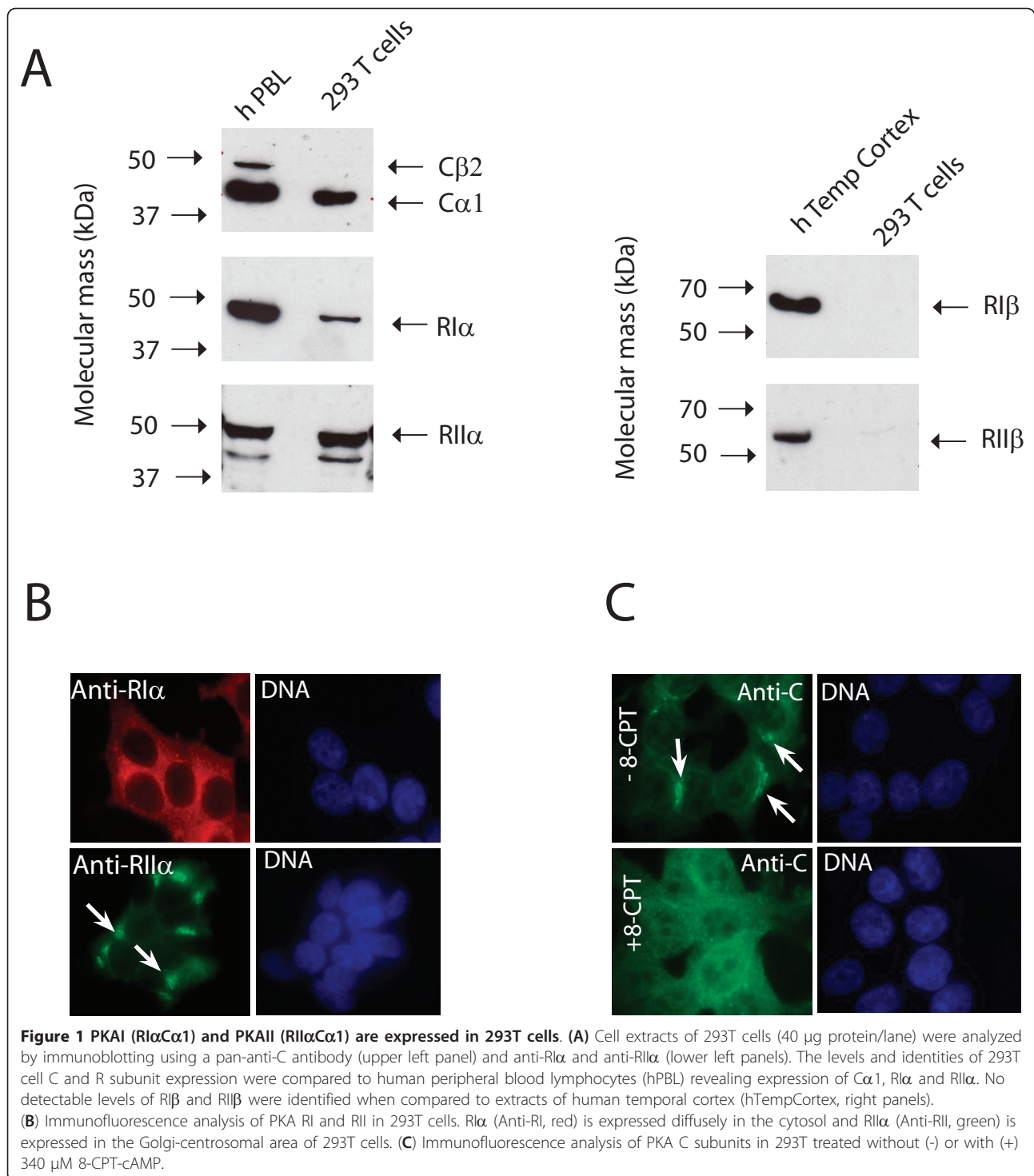
cAMP responsive element regardless of whether the holoenzyme contained  $\text{C}\alpha 1$  or  $\text{C}\beta 2$ . Our results contribute to understand the functional significance of two PKA holoenzymes but not various C subunits expressed in the same cell.

## 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  $\text{RI}\alpha$  and  $\text{RII}\alpha$  associated with  $\text{C}\alpha 1$  (Figure 1A, left panel), and not  $\text{RI}\beta$  and  $\text{RII}\beta$  (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- $\text{RI}\alpha$  (red) or anti- $\text{RII}\alpha$  (green) (Figure 1B). Co-immunostaining with anti-C demonstrated that  $\text{C}\alpha 1$  localization corresponded to R 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  $\mu\text{M}$ ) 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.

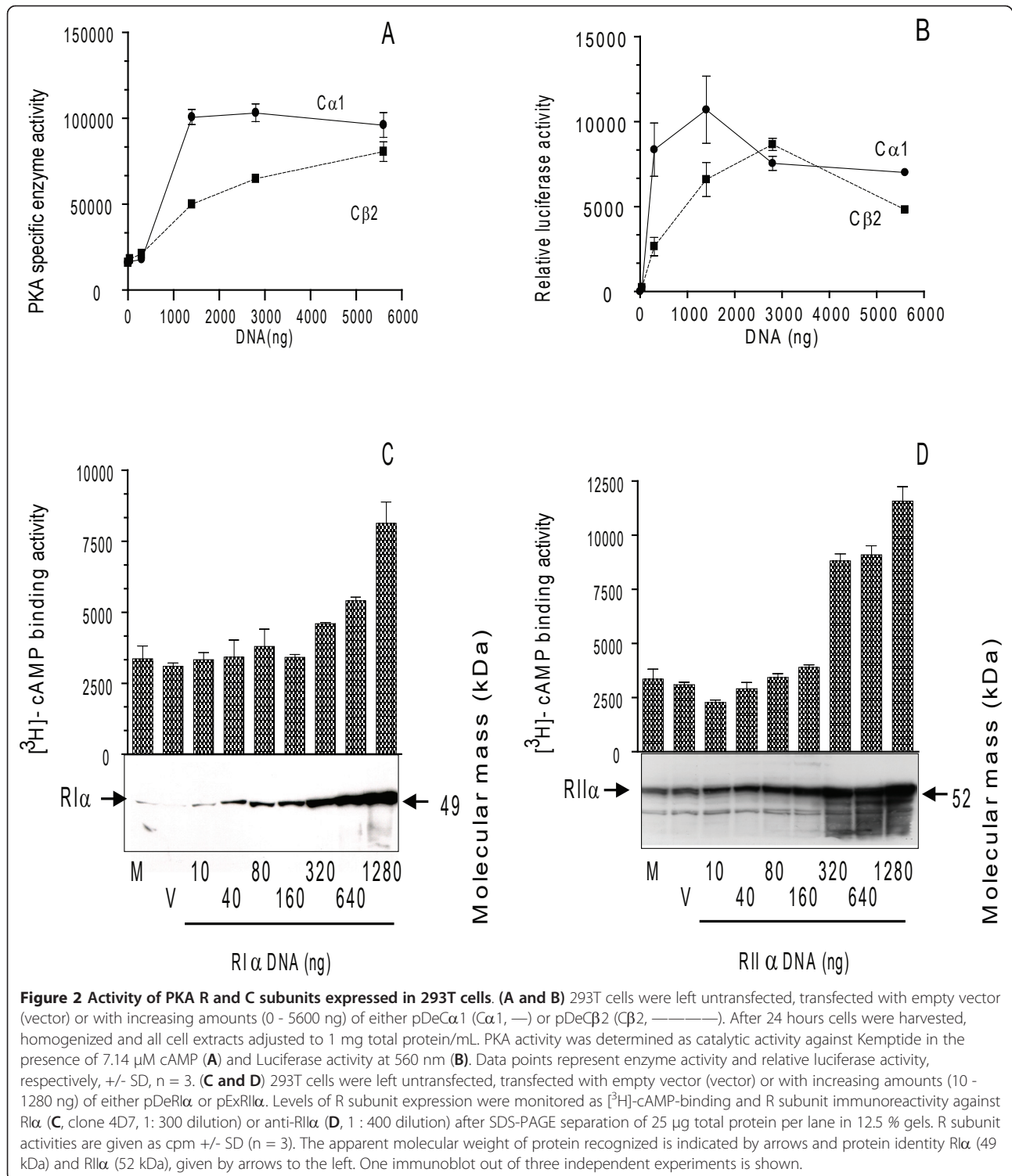
To obtain 293T cells dominated by either PKAI or PKAII expression, we formed holoenzymes by transient transfection of plasmids over-expressing either  $\text{RI}\alpha$  or  $\text{RII}\alpha$  (pDe $\text{RI}\alpha$  or pEx $\text{RII}\alpha$ ) in combination with either  $\text{C}\alpha 1$  or  $\text{C}\beta 2$  (pDe $\text{C}\alpha 1$  or pDe $\text{C}\beta 2$ ). 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 pDe $\text{C}\alpha 1$  and pDe $\text{C}\beta 2$  with a maximum at 5600 ng DNA (Figure 2A). The luciferase response was bell shaped and reached a maximum for pDe $\text{C}\alpha 1$  and pDe $\text{C}\beta 2$  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 pDe $\text{RI}\alpha$  and pEx $\text{RII}\alpha$ , respectively (Figure 2C, D).

Twenty four hours after transfection cells were lysed and R subunit levels were measured by immunoblotting and [ $^3\text{H}$ ]-cAMP-binding. This revealed an increase in a 49 kDa immunoreactive band as well as increased [ $^3\text{H}$ ]-cAMP-binding that coincided with the amount of plasmid transfected (pDe $\text{RI}\alpha$ , Figure 2C). The same was the case when transfecting pEx $\text{RII}\alpha$  (Figure 2D). Together this demonstrated a dose-dependent expression of both  $\text{RI}\alpha$  and  $\text{RII}\alpha$ .



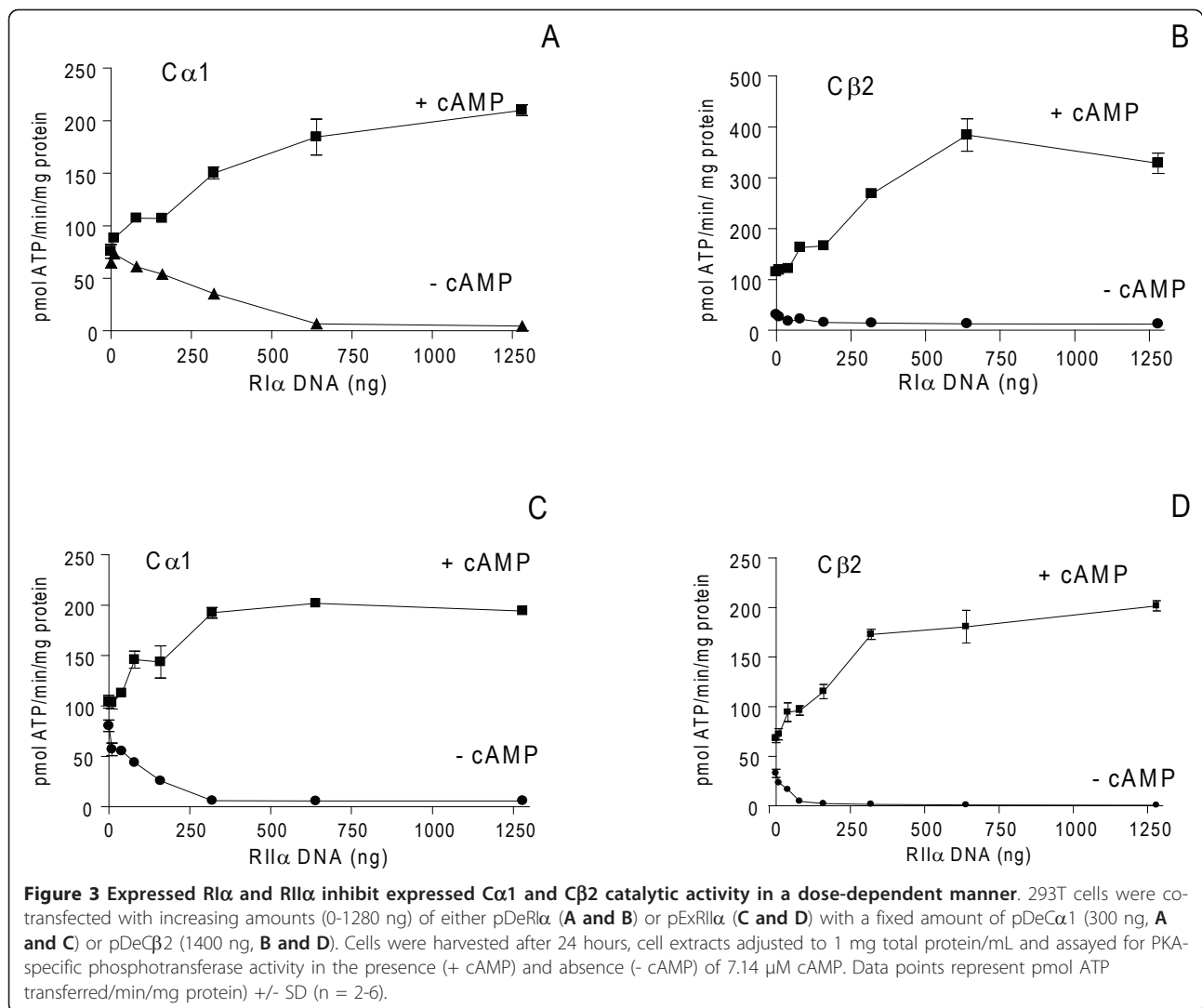
Based on these transfections and earlier experiments (results not shown), we next formed PKA holoenzymes by R and C co-transfections. We aimed at transfecting R plasmids to levels where C activity in the absence of cAMP were at basal levels, implying levels of R able to associate with all C subunits. 293T cells were co-

transfected with a fixed amount of either pDeCα1 (300 ng) or pDeCβ2 (1400 ng) together with increasing amounts of pDeR1α (0-1280 ng, Figure 3A, B) and pEx-R11α (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 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 pDeRIα (Figure 3A), which was equal to 28 ± 1.4 pmol RIα/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 RIα/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 pExRII $\alpha$  was required to inhibit C $\beta$ 2 activity to basal levels (Figure 3D) which was equal to  $9.6 \pm 2$  pmol RII $\alpha$ /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 pDeC $\alpha$ 1 or 1400 ng pDeC $\beta$ 2 and increasing amounts of pDeRI $\alpha$  and pExRII $\alpha$  (160-1280 ng DNA, Figure 4A-D). In these experiments psv- $\beta$ -Galactosidase (1000 ng) was

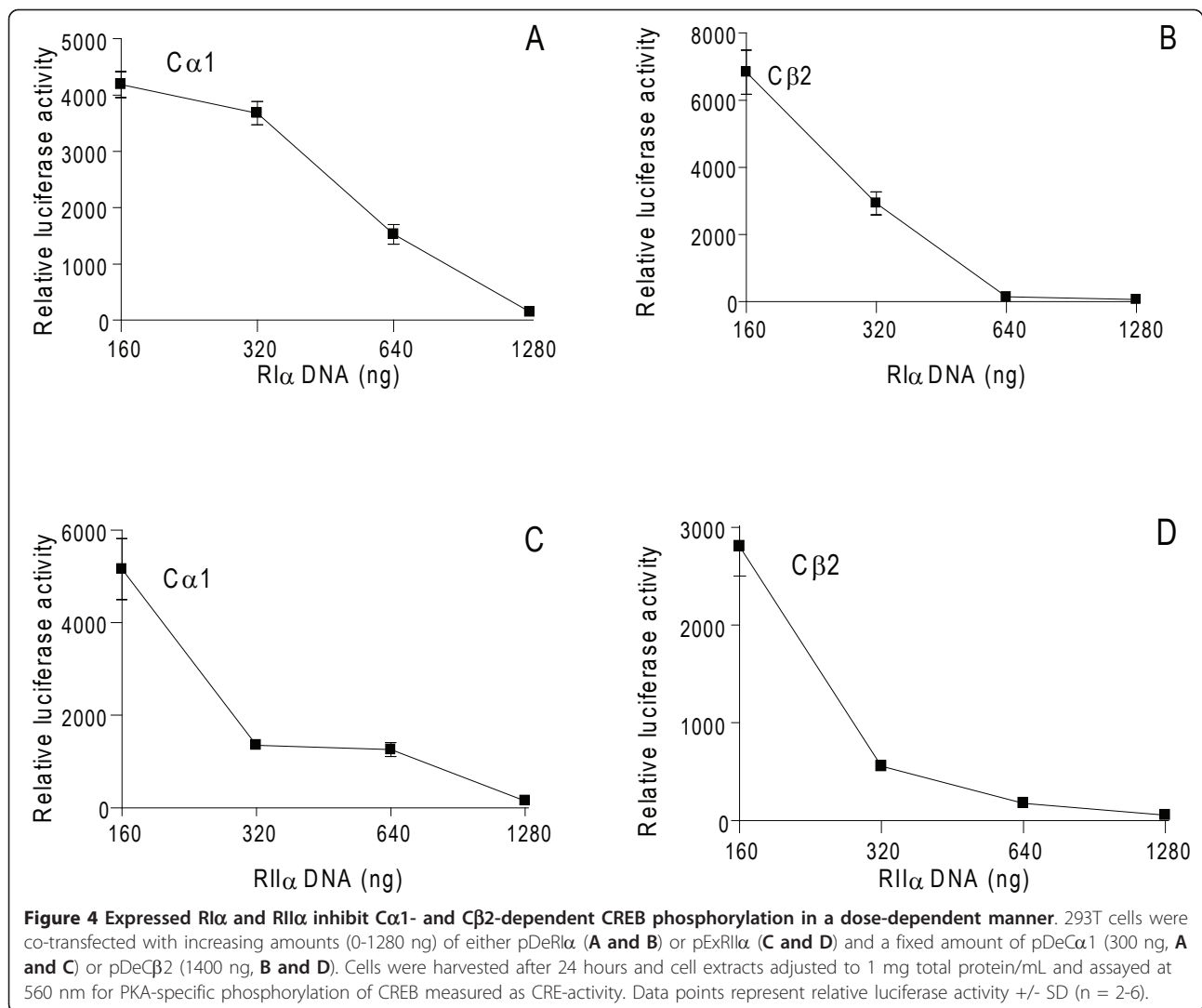
used for normalization (see Methods). This showed that luciferase activity induced by C $\alpha$ 1 and C $\beta$ 2 was completely inhibited by both RI $\alpha$  and RII $\alpha$  at doses above or equal to 640 ng plasmid DNA.

The experiments in Figures 3 and 4 depict that C $\beta$ 2 activity is fully inhibited at lower amounts of R than C $\alpha$ 1 is. This may imply that C $\alpha$ 1 is enzymatically more active than C $\beta$ 2 or simply that C $\beta$ 2 is more unstable than C $\alpha$ 1 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 C $\alpha$ 1 and C $\beta$ 2 display differential stability, identical amounts of C $\alpha$ 1 and C $\beta$ 2 plasmids were transfected alone or with 1280 ng of pDeRI $\alpha$ . This confirmed (Figure 5 bars 2 and 3) that in the absence of RI $\alpha$  total C $\beta$ 2 activity is significantly (\* p < 0.05) lower compared to C $\alpha$ 1. This was not the case when RI $\alpha$  was co-transfected with the two C subunits. In this case both C $\alpha$ 1 and C $\beta$ 2 activities were

**Table 1 Concentrations of RI and RII required for maximal inhibition of transfected C subunit**

Subunits	C $\alpha$ 1 (300 ng DNA)	C $\beta$ 2 (1400 ng DNA)
RI $\alpha$	$28 \pm 1.4^*$	$11.8 \pm 2.7$
RII $\alpha$	$16.2 \pm 0.5$	$9.6 \pm 2$

\*Concentration of R subunit (pmol/mg protein) required for 100% inhibition of C $\alpha$ 1 and C $\beta$ 2 activity.

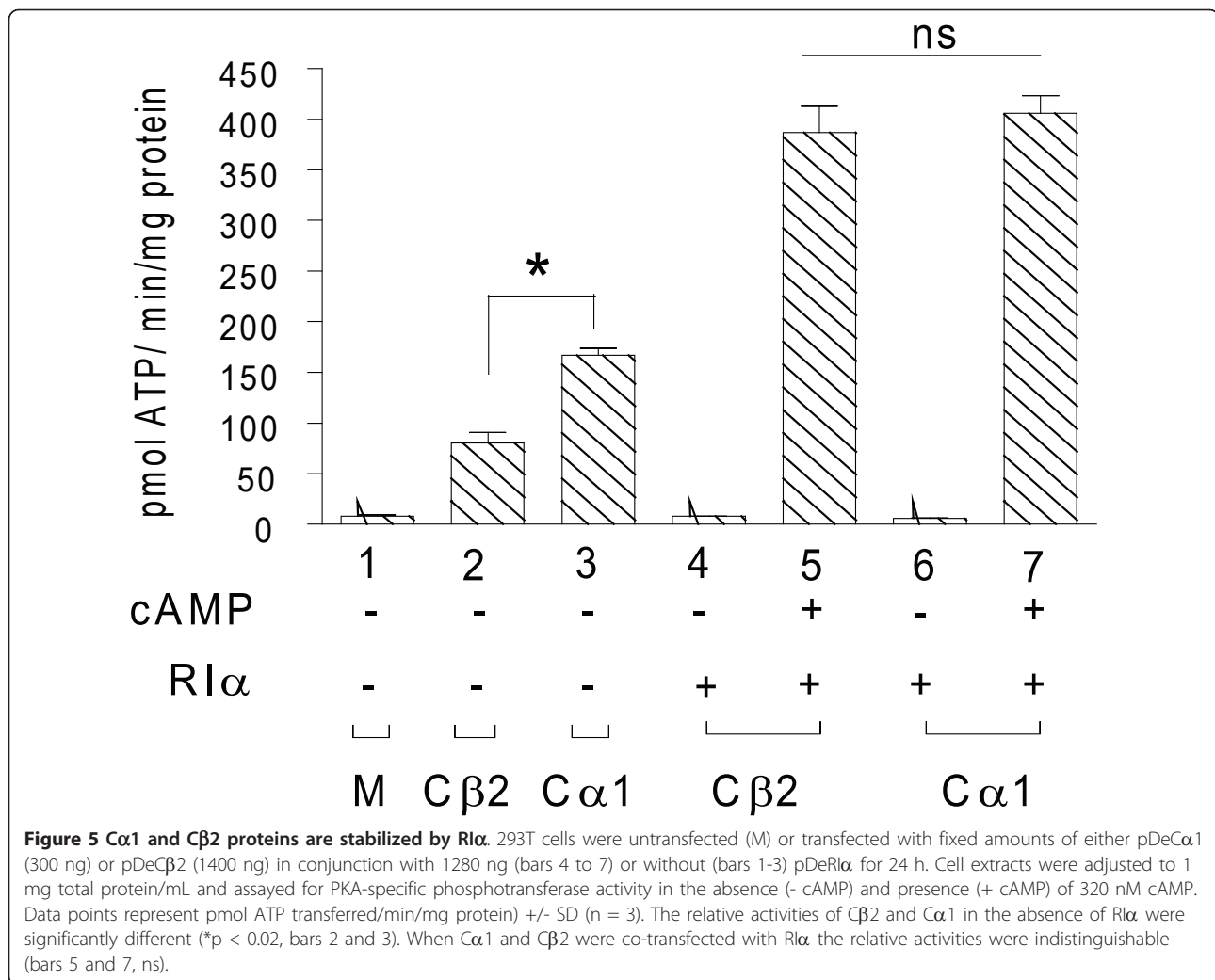


increased, however, to comparable levels after stimulation with cAMP (bars 5 and 7, ns). This demonstrated that R1 $\alpha$  has a stabilizing effect on both C subunits. However the effect was more pronounced for C $\beta$ 2 than C $\alpha$ 1 indicating that C $\beta$ 2 is more unstable than C $\alpha$ 1 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 R1 $\alpha$  and RII $\alpha$  required for complete inhibition of C $\alpha$ 1 and C $\beta$ 2 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 R1 $\alpha$  and RII $\alpha$  were able to inhibit C $\alpha$ 1 and C $\beta$ 2 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

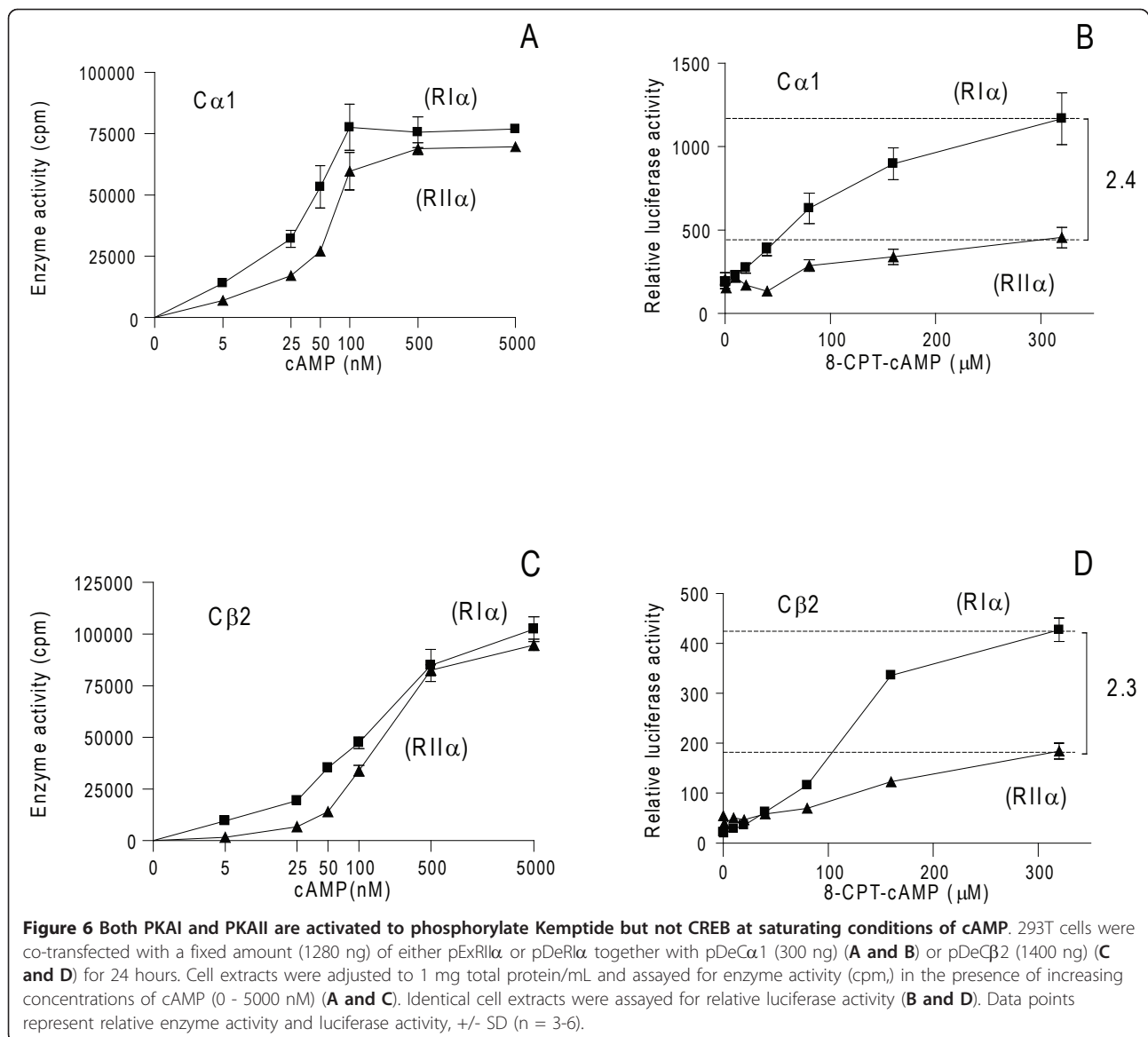
case of C $\alpha$ 1 at 100 nM cAMP when co-expressed with R1 $\alpha$  and between 500 and 5000 nM when co-expressed with RII $\alpha$ . In the case of C $\beta$ 2, maximum activity was achieved at concentrations between 500 and 5000 nM cAMP when co-expressed with both R1 $\alpha$  and RII $\alpha$ . 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  $\mu$ M) for 1 hour prior to harvesting. We observed that activity associated with C $\alpha$ 1 and C $\beta$ 2 released from both R1 $\alpha$  and RII $\alpha$  increased in a dose-dependent manner, reaching maximum between 160 and 320  $\mu$ M 8-CPT-cAMP (Figure 6B, D). However, a more than two fold higher activity was observed against CREB when C $\alpha$ 1 and C $\beta$ 2 were released from R1 $\alpha$  than from RII $\alpha$ . 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 pDeRIα (640 ng) and pExRIIα (320 ng) with Cα1 (300 ng pDeCα1) and monitored [<sup>3</sup>H]-cAMP binding. This showed equal activities (Figure 7A) and hence comparable levels (Table 2) 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 pExRIIα (320 ng) alone or in conjunction with pDeCα1 (300 ng). Transfected cells were treated without (0) or with incremental doses (1-320 μM) of 8-CPT-cAMP for four hours before harvesting. Equal amounts of cell extracts (50 μg total protein per lane) were analyzed for proteins immunoreactive to anti-RIα and anti-RIIα, respectively. Figure 7C shows that 8-CPT-cAMP



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  $\mu$ M) for 1 hour before harvesting. As depicted in Figure 7D 320  $\mu$ M 8-CPT-cAMP induced more than a 13-fold increase in luciferase activity when associated with RI $\alpha$  compared to untreated cells. When associated with RII $\alpha$  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,  $p < 0.01$ ).

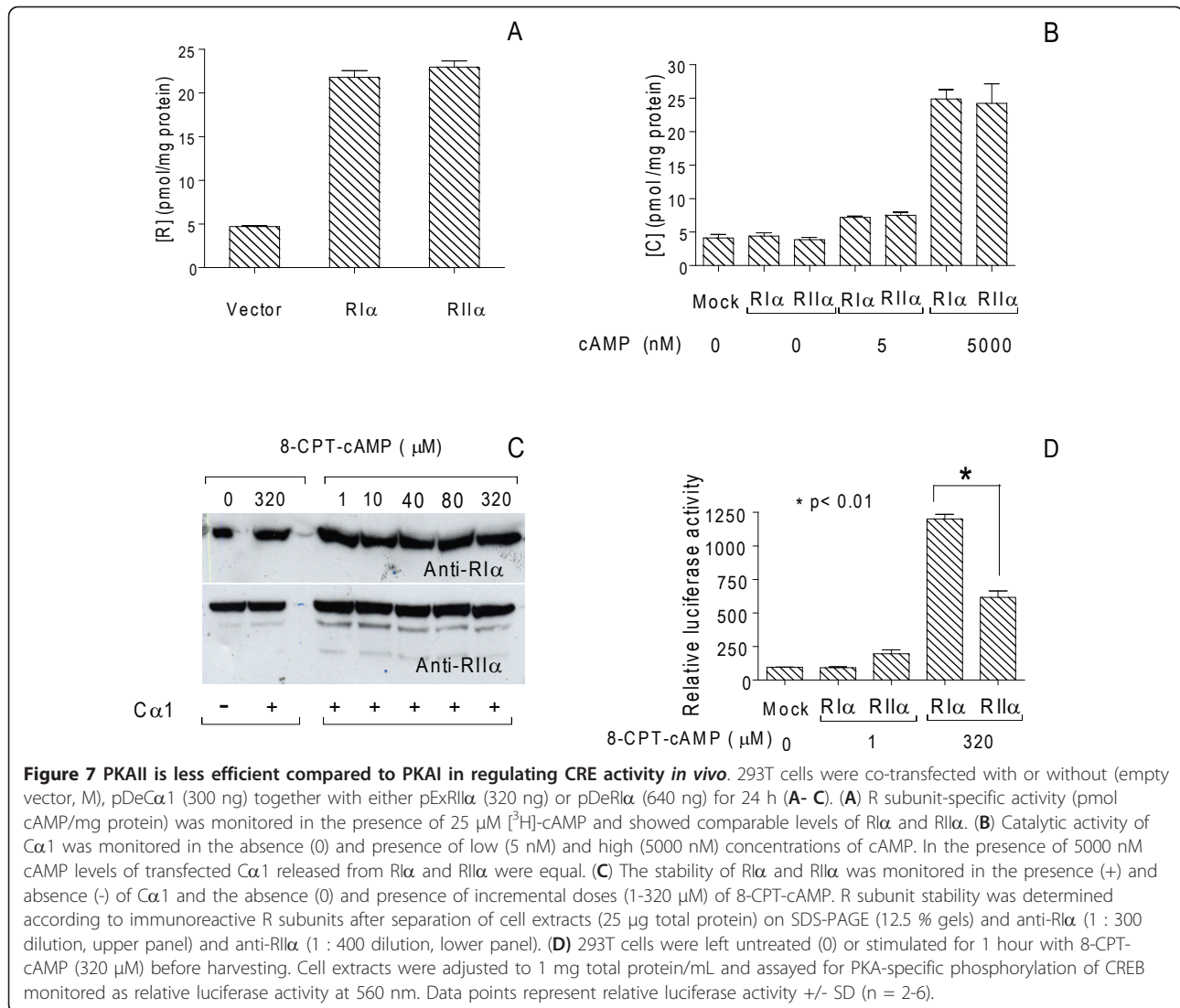
## Discussion

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 RI $\alpha$  or RII $\alpha$  together with either C $\alpha$ 1 or C $\beta$ 2.

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

**Table 2 Ratios of transfected R and C subunits**

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 [65].

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  $C\alpha 1$  was more active than  $C\beta 2$  when expressed in non-holoenzyme form. This may suggest differential  $K_d$  of  $C\alpha 1$  and  $C\beta 2$  against RI and RII. This suggestion was supported in that the amount of R plasmid required for complete inhibition of  $C\alpha 1$  and  $C\beta 2$ , respectively, was higher for RI compared to RII regardless of C subunit identity (28 pmol RI $\alpha$ /mg protein and 15 pmol RII $\alpha$ /mg protein for  $C\alpha 1$  versus ~12 pmol RI $\alpha$ /mg protein and ~10 pmol RII $\alpha$ /mg protein for  $C\beta 2$ ). However, we also observed that  $C\beta 2$ , but not  $C\alpha 1$  activity was stabilized when co-transfecting with the R subunit implying that the differences observed is due to protein instability of the  $C\beta 2$  subunit and not lower  $K_d$  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  $C\alpha 1$  and  $C\beta 1$  have identical  $K_d$  values for RI [55]. To what extent  $C\beta 2$  is more sensitive to proteasome degradation than  $C\alpha 1$  is not known. It should however be noted that the marked differences between the  $C\alpha 1$  and  $C\beta 2$  at the N-terminus has been implicated in C subunit stability. For  $C\alpha 1$  it has been demonstrated that the  $\alpha$ -helix and Trp 30 are vital moieties for  $C\alpha 1$  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  $C\beta 2$  versus  $C\alpha 1$  in inducing Cre-luciferase activity irrespective of association with RI $\alpha$  or RII $\alpha$ . The latter may suggest that  $C\alpha 1$  and  $C\beta 2$  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 as such as localization. The latter has previously been demonstrated in that the N-terminus of  $C\alpha 1$  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  $C\alpha 1$  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  $C\alpha 1$  to contribute to regulation and tuning of subcellular targeting. Despite lack of experimental evidence the N-terminal amphiphatic  $\alpha$ -helix in  $C\beta 2$  has been proposed to function as a targeting domain for  $C\beta 2$  *in vivo* [20]. Despite the obvious differences between  $C\alpha 1$  and  $C\beta 2$  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  $C\beta 2$  appear more stable in the presence of R subunit than  $C\alpha 1$ .

## 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-Pyruvate (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  $0.7 \times 10^6$  cells per well containing 1.5 mL RPMI medium without Penicillin/Streptomycin. Plates were kept at 37°C in a humidified atmosphere under 5% CO<sub>2</sub>.

### Generation and expression of PKA vectors

pEF-DEST 51<sup>TM</sup>(Invitrogen) expression vectors encoding human regulatory and catalytic subunits RI $\alpha$ , C $\alpha$ 1 and C $\beta$ 2 were created using Gateway LR Clonase Reaction<sup>®</sup> (Invitrogen) and transformed into Library<sup>®</sup> efficiency DH5 $\alpha$ <sup>TM</sup>Competent cells (Invitrogen). Plasmid pBlue-script containing RII $\alpha$  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<sup>®</sup> DH5 $\alpha$ <sup>TM</sup>Competent cells (Invitrogen). Plasmids expressing catalytic subunits C $\alpha$ 1 or C $\beta$ 2 or/and regulatory subunits RII $\alpha$  or RI $\alpha$  hereby termed pDeC $\alpha$ 1, pDeC $\beta$ 2, pDeRI $\alpha$ , and pExRII $\alpha$  where transfected using Lipofectamine 2000 (Invitrogen). In order to facilitate a reporter system, plasmids expressing Luciferase reporter gene and  $\beta$ -Galactosidase as a normalization control was co-transfected with R subunit and/or C subunit in constant amounts (0.7  $\mu$ g Cage-Cre-Luciferase reporter vector and 1  $\mu$ g Psv- $\beta$ -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

Immuno blotting was performed as previously described [15]. Membranes were incubated with mouse monoclonal anti-RII $\beta$  (cat # 610625, BD Transduction laboratories) at 1:250 dilution, polyclonal rabbit anti-RI $\beta$  (cat # SC-907, Santa Cruz Biotechnology, Inc.), anti-RII $\alpha$  (cat # 612243, BD Transduction laboratories) at 1:400 dilution or mouse monoclonal anti-RI $\alpha$  (Clone 4D7, [65]) at 1:300 dilution. Immunoreactive proteins were detected with HRP-conjugated secondary antibodies (ICN Diagnostics) and SuperSignal<sup>®</sup> 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  $\mu$ M Coenzyme A (Boehringer), 530  $\mu$ M ATP (Boehringer), 470  $\mu$ M 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 [<sup>3</sup>H]-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 RI (Clone 4D7, [65]) and RII (cat # 612243, BD Transduction laboratories) were diluted (see figure legend). The anti-C antibodies were rabbit polyclonal anti-C $\alpha$  1:100 (cat # sc 903, Santa Cruz Biotechnology, Santa Cruz, CA).

### Statistics

Data are presented as means  $\pm$  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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### 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. SØ 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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