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The testis-specific $C\alpha 2$ subunit of PKA is kinetically indistinguishable from the common $C\alpha 1$ subunit of PKA

Maike M Vetter¹, Hans-M Zenn¹, Eva Méndez², Heidrun van den Boom¹, Friedrich W Herberg¹ and Bjørn S Skålhegg^{2,3*}

Abstract

Background: The two variants of the α -form of the catalytic (C) subunit of protein kinase A (PKA), designated $C\alpha 1$ and $C\alpha 2$, are encoded by the *PRKACA* gene. Whereas $C\alpha 1$ is ubiquitous, $C\alpha 2$ expression is restricted to the sperm cell. $C\alpha 1$ and $C\alpha 2$ are encoded with different N-terminal domains. In $C\alpha 1$ but not $C\alpha 2$ the N-terminal end introduces three sites for posttranslational modifications which include myristylation at Gly1, Asp-specific deamidation at Asn2 and autophosphorylation at Ser10. Previous reports have implicated specific biological features correlating with these modifications on $C\alpha 1$. Since $C\alpha 2$ is not modified in the same way as $C\alpha 1$ we tested if they have distinct biochemical activities that may be reflected in different biological properties.

Results: We show that $C\alpha 2$ interacts with the two major forms of the regulatory subunit (R) of PKA, RI and RII, to form cAMP-sensitive PKAI and PKAII holoenzymes both *in vitro* and *in vivo* as is also the case with $C\alpha 1$. Moreover, using Surface Plasmon Resonance (SPR), we show that the interaction patterns of the physiological inhibitors RI, RII and PKI were comparable for $C\alpha 2$ and $C\alpha 1$. This is also the case for their potency to inhibit catalytic activities of $C\alpha 2$ and $C\alpha 1$.

Conclusion: We conclude that the regulatory complexes formed with either $C\alpha 1$ or $C\alpha 2$, respectively, are indistinguishable.

Keywords: PKA, Catalytic subunit, N-terminal, splice variants

Background

Cyclic 3', 5'-adenosine monophosphate (cAMP) is a key intracellular signaling molecule, whose main function is to activate the cAMP-dependent protein kinase (PKA) [1]. PKA is a heterotetrameric holoenzyme consisting of a regulatory (R) subunit dimer and two catalytic (C) subunits. The holoenzyme is activated when four molecules of cAMP bind to the R subunit dimer, two to each R subunit, releasing two free active C subunits [2;3]. In man, four different R subunits designated RI α , RI β , RII α , RII β (reviewed in [4]), and four different C subunits ($C\alpha$, $C\beta$, $C\gamma$ and PrKX) have been identified [3]. The $C\alpha$ and $C\beta$ subunits are expressed in most tissues, while the

$C\gamma$ subunit, which is transcribed from an intron-less gene, represents a retroposon derived from the $C\alpha$ subunit [5]. $C\gamma$ is only expressed in human testis [6]. PrKX is an X chromosome-encoded protein kinase, and was identified as a PKA C subunit since it is inhibited by both PKI and RI α , and the RI α /PrKX complex is activated by cAMP [7].

Splice variants of both $C\alpha$ and $C\beta$ have been identified. In the case of $C\alpha$, two splice variants have been identified and termed $C\alpha 1$ [8] and $C\alpha 2$ [9-11]. $C\alpha 1$ and $C\alpha 2$ have non-identical N-terminal ends encoded by alternative use of two exons (1a and 1b, mouse terminology) located upstream of exon 2 in the murine $C\alpha$ gene. The $C\beta$ gene encodes a number of products identified in various species and have been designated $C\beta 1$, $C\beta 2$, $C\beta 3$, $C\beta 4$, $C\beta 3ab$, $C\beta 3b$, $C\beta 3abc$, $C\beta 4ab$, $C\beta 4b$, $C\beta 4abc$ [12-17]. As is the case for the $C\alpha$ forms, all the

* Correspondence: b.s.skulhegg@medisin.uio.no

²Department of Nutrition, Institute of Basic Medical Sciences, University of Oslo, Pb 1046 Blindern, 0316 Oslo Norway

Full list of author information is available at the end of the article

C β variants have variable N-terminal ends which are encoded by different exons upstream of exon 2 in the C β gene [14;15].

PKA-C splice variants are tissue-specifically expressed and some experimental evidence support that they may harbor specific features and non-identical activities when associated with the R subunits to form holoenzymes [18;19]. With regard to this C α 2 it is the sole C subunit expressed in the sperm cell. Moreover, C α 2 was shown to be vital for mouse sperm motility since ablation rendered the sperm cells non-motile and the male individuals infertile [9-11;20].

C α 1 is equipped with an N-terminal of 14 amino acids which undergo three well defined co- and posttranslational modifications. They include *in vivo* myristylation of Gly1 [21]. At position +1 an Asn is encoded which is partly deamidated *in vivo* leading to C α 1-Asp2 and C α 1-iso(β)Asp2 [22]. A third modification is identified as a PKA-autophosphorylation site at Ser10 [23-25]. C α 2 on the other hand is encoded with 7 unique amino acids at the N-terminus which to our knowledge do not have the ability to undergo any of the N-terminal modifications seen for C α 1.

Based on the different N-terminal sequences of C α 1 and C α 2 we speculate that they will introduce distinct biological features to these subunits. To investigate this hypothesis we made a thorough characterization of C α 2 activities both *in vivo* and *in vitro* and compared the results to what is known for C α 1 and to results obtained for C α 1 in the present work.

Methods

Sperm cell isolation

Semen samples were obtained from patients attending infertility investigations at the Andrology Laboratory at Rikshospitalet-Radiumhospitalet HF, Oslo, Norway. All patients signed a letter of approval and all experiments were done according to the recommendation from the Regional Committees for Medical and Health Research Ethics. All men produced their ejaculates on site or at home after 3-5 days of sexual abstinence. Samples were collected by masturbation into a wide-mouthed sterile container (Sarstedt Ltd., Leicester, United Kingdom) and after 30 min of liquefaction at 37°C, sperm parameters were evaluated according to World Health Organization (WHO) methods (World Health Organization, WHO laboratory manual for the examination of human semen and sperm-cervical mucus interaction (4th ed.), Cambridge University Press, Cambridge (1999)).

Sperm cells were isolated from the seminal plasma by percoll gradient centrifugation. Sperm samples were pipetted on top of a 90%/45% percoll gradient and centrifuged at 2500 rpm for 20 min, no brake. After centrifugation, the sperm pellet was recovered by first using a

sterile glass Pasteur pipette to remove the top layers of the semen sample and sperm gradient, leaving approximately 0.5 mL of the bottom layer. The sperm pellet was subsequently resuspended and washed twice in phosphate buffered saline (PBS) and centrifuged again at 2500 rpm for 8 min.

Sperm head and tail separation

Isolated sperm cells were diluted to 1 mill/mL in PBS and sonicated mildly for 10 sec at low frequency. Ten μ L samples were taken out to be examined by microscopy to assure head and tail separation. After complete separation the mixture was centrifuged at 400 g for 10 min. The supernatant containing the tails was transferred to a new tube and tails pelleted by centrifugation at 10.000 \times g for 15 min. The tail pellet and the pellet from the first centrifugation were separately solubilized in RIPA buffer (10 mM Tris-HCl pH 7.5, 1 mM EDTA, 1% Triton X-100, 0.1% SDS, 0.1% Na-deoxycholate and 100 mM NaCl) containing 1 mM DTT, 1 mM PMSF and protease inhibitor cocktail (Roche), and sonicated 2 \times 10 seconds at full effect.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE, was performed as described by [26]. Briefly, samples were diluted in SDS sample buffer (62.5 mM Tris-HCl, pH 6.8, 2.3% SDS, 10% glycerol, 5% β -mercaptoethanol, 0.001% bromophenol blue), boiled for 2 min and loaded onto slab gels consisting of a 4.5% stacking gel and a 12.5% separating gel.

Immunoprecipitation

Lysates were cleared by centrifugation at 15000 g for 30 min at 4°C, and subsequently incubated with primary antibody [anti-C α 2 (SNO101; 320 μ g/mL), mouse anti-R1 α (2.5 μ g/mL), rabbit anti-R11 α serum diluted 1:100] for 2 h to overnight. Antibody-antigen complexes were precipitated using either Dynabeads protein G (Dyna, catalogue number 100.04), anti-mouse agarose beads or anti-rabbit agarose beads (Sigma, catalogue number A6531, A1027). Precipitates were washed three times using appropriate buffer and extracted with buffer in the presence or absence of 1 mM 8-CPT cAMP as indicated in the figure legends.

Immunoblot analysis

Total protein was estimated by Bradford protein assay (BioRad). Proteins were separated by SDS-PAGE and transferred to PVDF membranes by electro blotting. Membranes were blocked in 5% skimmed milk powder in Tris-buffered saline containing 0.1% Tween-20 (TBST) for 1 h at room temperature, and then incubated for 1 h at room temperature or overnight at 4°C with the appropriate primary antibodies diluted in TBST

rabbit polyclonal anti-C 1:1000 (Santa Cruz Biotechnology), anti-C α 2 (SNO101), mouse anti-RI α 1:250 and rabbit anti-RII α serum [27]. Membranes were washed for about 1 h in TBST and further incubated with HRP-conjugated secondary antibodies (ICN Diagnostics). Membranes were washed and finally developed using SuperSignal[®] West Pico Chemiluminescent (Pierce).

Endogenous sperm cell protein fractionation

Sperm cells were homogenized in PE buffer (5 mM KH₂PO₄, 5 mM K₂HPO₄, 1 mM EDTA, pH 6.8) containing 0.5% Triton X-100, 1 mM DTT, 1 mM PMSF and protease inhibitor cocktail (Roche), and sonicated 2 × 10 seconds. DEAE cellulose (Whatman DE 52) was applied to a column and equilibrated with PE buffer with 250 mM sucrose. The sperm cell homogenates were cleared by centrifugation at 15.000 g for 30 min at 4°C and applied to the column. After washing with PE buffer, the column was eluted with a linear 0-400 mM NaCl gradient created by a gradient mixer (total volume 50 ml), 1 ml fractions were collected and salt concentration checked by refractometry. Every other fraction, starting with fraction 1 was subjected to phosphotransferase activity measurements in the absence and presence of cAMP. Peak fractions were concentrated to 100 μ l using centrifugal filters with 30 kDa cutoff (Millipore) and subjected to immunoblot analysis.

Phosphotransferase assay

Catalytic activity of PKA was assayed by phosphorylating the PKA specific substrate Kemptide (Peninsula) using γ -[³²P]ATP (5000 mCi/ μ mol) as previously described [26].

Specific activity is determined as U/mg, which is defined as μ mol/mg × min.

cAMP-binding assay

Determination of specific cAMP-binding of soluble R subunits was carried out in a buffer containing 0.3 μ mol [³H]-cAMP (spec. act. 41.7 Ci/mmol, Amersham) [27].

Activation Assay

The activation assay of C α 2 and myrC α 1 with RI α and RII β , respectively, was performed in a spectrophotometric kinase activity assay as described earlier [28].

Expression of C α 2, C α 1 and myristylated C α 1

Recombinant non-myristylated human C α 1 was expressed and purified as described previously [29;30]. Recombinant human C α 1, as well as recombinant human C α 2, were co-transformed with N-myristyl-transferase in Escherichia coli BL21 (DE3) (Novagen) and co-expressed with human C α 1 as well as C α 2 using the same conditions. Both proteins were purified by affinity chromatography using PKI-peptide Affi-Gel. The

procedure was first described by Olsen *et al.* [31] and modified after Thullner *et al.* [32].

Expression and purification of R subunits

Recombinant human R subunits (hRI α , hRI β , hRII α , hRII β) were over-expressed in Escherichia coli BL21 (DE3) RIL (Novagen) and purified according to a procedure by Bertinetti *et al.* [33] using Sp-8-AEA-cAMPS-agarose.

The purity of the R and C proteins was confirmed by SDS-PAGE as well as by immunoblot analysis and the biological activity of the proteins was measured as described before [30]. Primary sequence of C α 2 and the presence of N-myristylation at C α 1 were checked by mass spectrometry (Data not shown).

SPR analysis

All SPR interaction analyses were performed at 25°C in 20 mM MOPS pH 7, 150 mM NaCl plus 0.005% (v/v) surfactant P20, 1 mM ATP, 5 mM MgCl₂ and 50 μ M EDTA using Biacore 2000 or 3000 instruments (GE Healthcare-Biacore, Sweden). For covalent coupling of the C subunits, carboxymethylated sensor chip surfaces (CM5, research grade, GE Healthcare) were activated with NHS/EDC for 7 min and non-myristylated C α 1, C α 2 and myrC α 1 (5 μ g/ μ l in 10 mM sodium acetate plus 200 μ M ATP and 500 μ M MgCl₂ with a pH 6.0) as described [34] were injected on separate flow cells with a flow rate of 5 μ l/min until approximately 300 response units (RU 1,000 RU = 1 ng/mm² for a CM 5 chip) [35] were reached. This amine coupling was described previously [36;37]. Deactivation of the surface was performed using 1 M ethanolamine-HCl (pH 8.5) for 7 min. As a reference one flow cell was activated and deactivated in the absence of any protein.

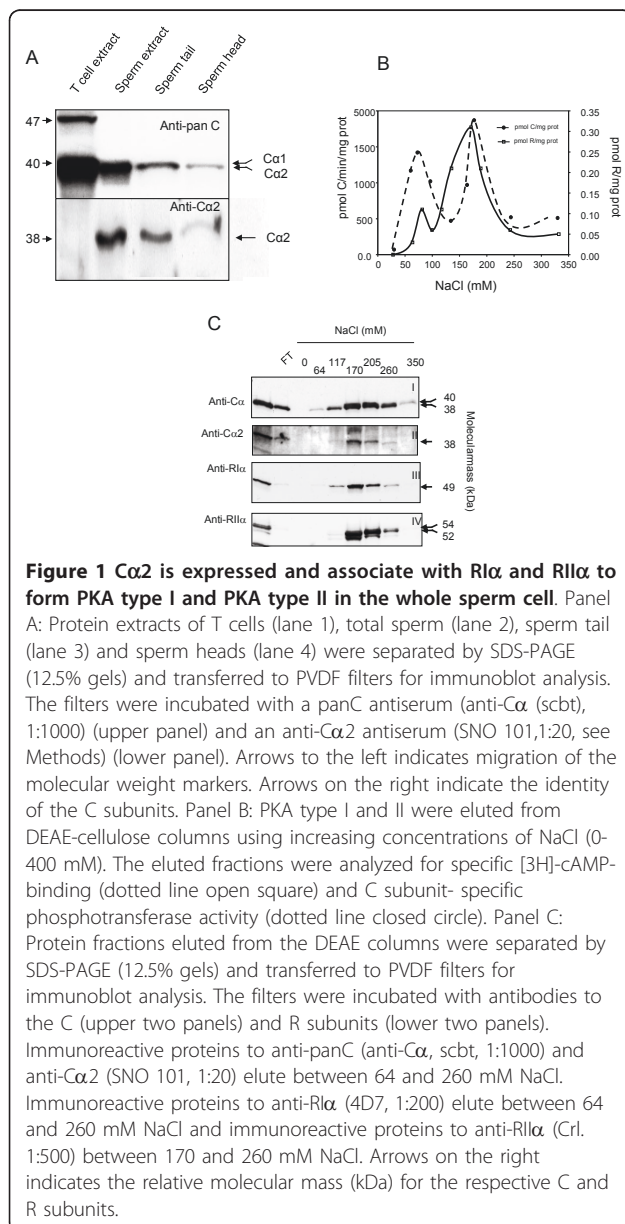
The interaction experiments with four R subunit were performed at 25°C in running buffer (20 mM MOPS pH 7, 150 mM NaCl, 1 mM ATP, 5 mM MgCl₂, 50 μ M EDTA, 0.005% surfactant P20) at a flow rate of 30 μ l/min. During simultaneous R subunit injection over the four surfaces (150 sec), the dissociation phase was monitored for 150 sec. The binding of R subunit to C subunit was not limited by mass transport according to previous experiments [38]. Response of the activated/deactivated reference cell was subtracted. Surfaces were regenerated with two subsequent 1 min injections of 0,1 mM cAMP, 2,5 mM EDTA in running buffer. Evaluation of non-normalized data was performed with Biaevaluation 3.2 RC1 (GE Healthcare). A Langmuir 1:1 binding model was applied for the kinetic analysis of C subunit/R subunit interactions [37].

The sensor chip was stored at 4°C in running buffer and tested for proper performance prior to each analysis.

Results

Sperm cell-specific $C\alpha 2$ associates with $R I\alpha$ and $R I I\alpha$ in a cAMP-dependent fashion to form PKAI and PKAII holoenzymes in vivo

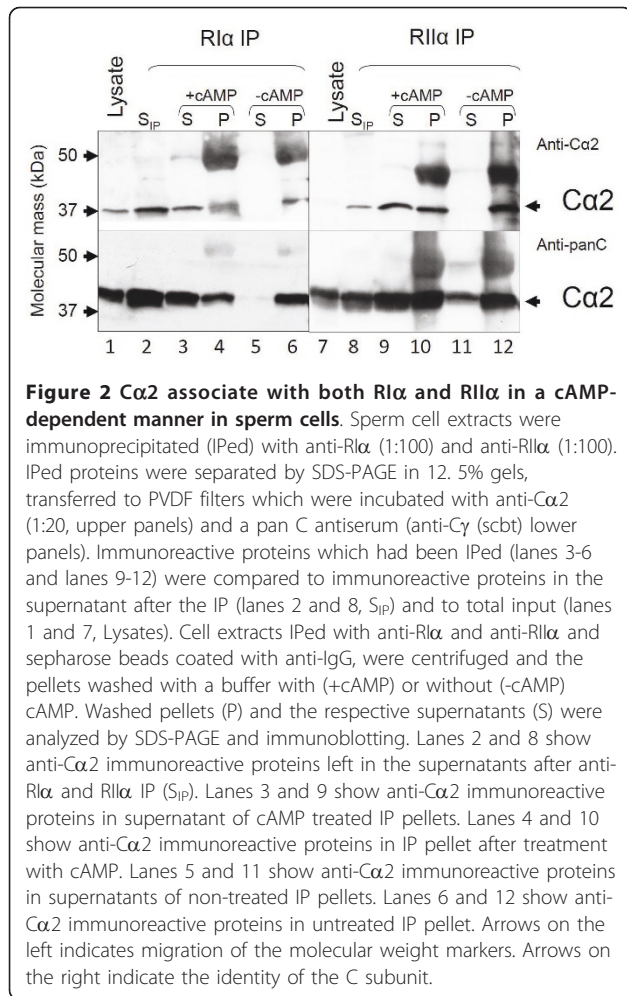
We first determined whether human sperm cells express $C\alpha 2$. Protein extracts of T cells, whole sperm, sperm tail, and sperm heads were separated by SDS-PAGE transferred to immunoblot filters and filters probed with a pan-C antibody (Figure 1A, left panel, anti-C) and an anti- $C\alpha 2$ antiserum (SNO 101, anti- $C\alpha 2$ [10], Figure 1A, right panel). This confirms previous results that $C\alpha 2$ is not expressed in T cells and is distributed in tail and head of spermatozoa [10]. RI and RII are both expressed in all sperm cell compartments and are



known to form PKA type I and type II (PKAI and PKAII) [39-41]. PKAI and PKAII holoenzymes can be separated by DEAE ion exchange chromatography using increasing concentrations of NaCl [42]. Since $C\alpha 2$ is the sole C subunit in human sperm cells it is expected that it will associate with RI and RII to form PKAI and PKAII, respectively, as has been demonstrated in the mouse [11]. To test this, whole sperm cell extracts (3 mg) were fractionated on DEAE resins by a linear NaCl gradient ranging from 0 to 350 mM). Two peaks of phosphotransferase- (—●—) and cAMP-binding activity (—□—) between 50-100 mM and 100-250 mM NaCl, respectively, were observed. This implied formation of both PKAI and PKAII (Figure 1B). C and R subunit identity were documented by immunoblotting using a pan-C antibody (Figure 1, panel CI) and anti- $C\alpha 2$ (panel CII) as well as anti- $R I\alpha$ (panel CIII) and anti- $R I I\alpha$ (panel CIV). This showed that immunoreactive $C\alpha 2$ co-elutes with the two major R subunits in sperm cells, $R I\alpha$ (peak I) and $R I I\alpha$ (peak II) [43-45]. From the figure it is also seen that some of the C activity was detected before the R subunit activity in the first peak, implying free C subunit. Moreover, we also noted that PKAI and PKAII containing $C\alpha 2$ eluted at comparable concentrations of NaCl. To further investigate whether human $C\alpha 2$ forms PKAI and PKAII in cell extracts we immunoprecipitated (IPed) with anti- $R I\alpha$ and anti- $R I I\alpha$ (Figure 2). Using immunoblotting and anti- $C\alpha 2$ (upper panels) and anti-pan-C (lower panels) we showed that both $R I\alpha$ and $R I I\alpha$ associate with $C\alpha 2$, implying that $C\alpha 2$ forms PKAI and II *in vivo* (lanes 6 and 12). To define whether the R-C interaction is specific and functional, the IPed proteins were challenged with cAMP to dissociate the holoenzyme into R subunit dimers and free C subunits [46]. In these experiments the IPed R subunits would be expected to be immobilized by the precipitating antiserum and remain in the pellet (P) after the cAMP wash whereas the IPed C subunit would be released into the supernatant (S) in the presence of cAMP [46;47]. Figure 2 (lanes 3 and 4, and 9 and 10) depicts that immunoreactive $C\alpha 2$ is released into the supernatant after the cAMP wash (+cAMP) after both anti- $R I\alpha$ and anti- $R I I\alpha$ IP (upper and lower panels) whereas the immunoreactive $C\alpha 2$ subunit remained in the pellet (P) in the absence of cAMP (-cAMP) (Figure 2, lanes 5 and 6 and 11 and 12, upper and lower panels).

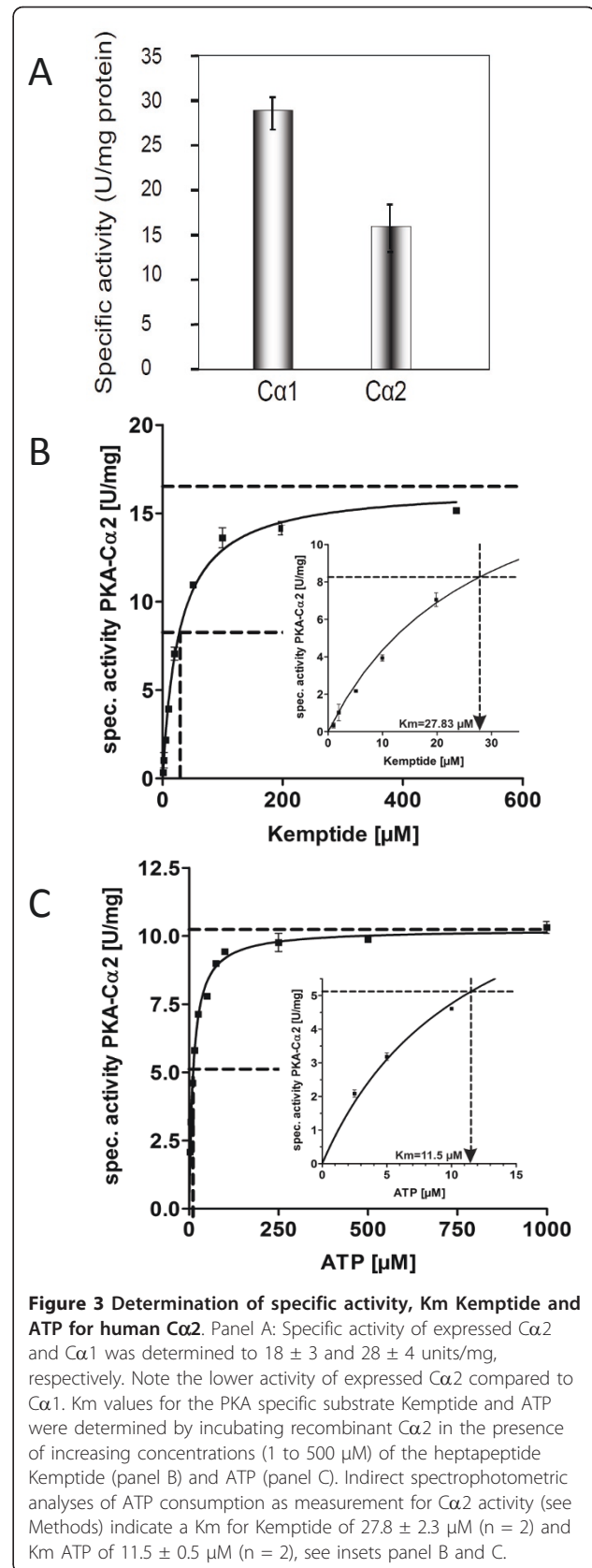
Comparable activities of $C\alpha 2$ and $C\alpha 1$

Our results and several reports showing that $C\alpha 2$ associates with both RI and RII subunits [3; 48-50] in a cAMP-sensitive fashion imply that N-terminal differences do not interfere with PKA holoenzyme formation.. To further investigate whether $C\alpha 2$ has activities that differ from $C\alpha 1$ we expressed $C\alpha 2$ using the pREST B



vector. First we noted that $\text{Ca}2$ and $\text{Ca}1$ were captured in the soluble and particulate fractions of the bacteria lysates, respectively, suggesting differences in solubility (results not shown). Furthermore, specific activity of expressed $\text{Ca}2$ was determined to 18 ± 3 units/mg (U/mg, $n = 3$) which was notably lower than the specific activity of expressed $\text{Ca}1$ (28 ± 4 U/mg, Figure 3A). Taken together this may imply differential features of $\text{Ca}1$ and $\text{Ca}2$. Based on this and to determine the exact activities for $\text{Ca}2$ three features were investigated. These included (i) $\text{Ca}2$'s substrate affinity, (ii) the ability to form holoenzymes with RI and RII *in vitro* and (iii) the K_m values for Kemptide and ATP. In the latter case we found the K_m values of $\text{Ca}2$ for Kemptide and ATP to be $27.8 \pm 2.3 \mu\text{M}$ and $11.5 \pm 0.5 \mu\text{M}$, respectively (Figure 3B and 3C). This is in good agreement with previous results obtained with expressed $\text{Ca}1$ [7].

We next monitored cAMP-sensitivity of type I and II PKA holoenzymes containing $\text{Ca}2$ *in vitro*. To calculate the accurate cAMP activation constant, PKAI ($\text{RI}\alpha$) and

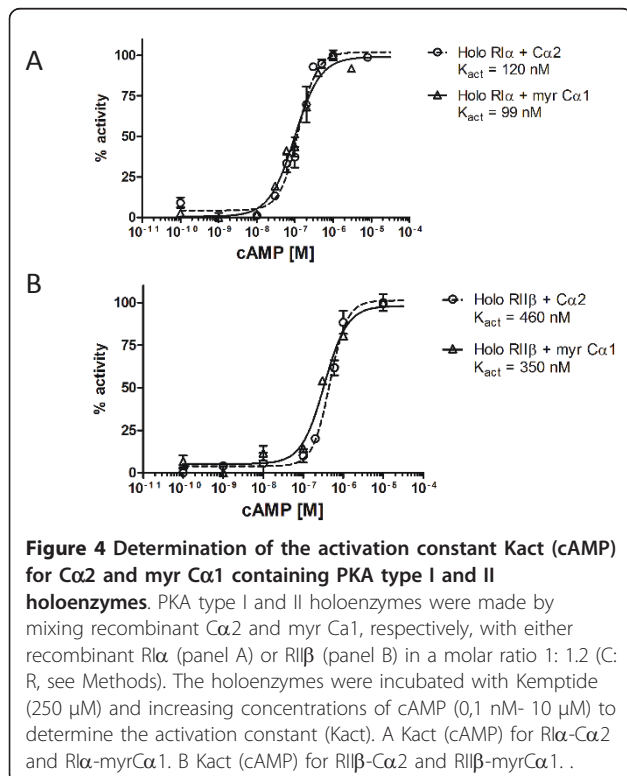


PKAII (RII β) containing $C\alpha 2$ holoenzymes were incubated with 250 μ M Kemptide and 10 mM ATP in the presence of increasing concentrations of cAMP. K_{act} values for cAMP were 120 nM and 460 nM for the holoenzyme formed with RI α and RII β , respectively, showing that PKAI containing $C\alpha 2$ are nearly 4 fold more sensitive to cAMP than PKAII containing $C\alpha 2$ holoenzymes (figure 4A,B). The corresponding values for holoenzymes formed with myr $C\alpha 1$ were 99 nM (RI α) and 350 nM (RII β) (figure 4A,B) again demonstrating a 4 fold increased sensitivity for the RI holoenzyme. This is also in agreement with previous *in vitro* results for PKAI and II holoenzymes containing $C\alpha 1$ [38;51], and the K_{act} values for mouse PKAI isolated from sperm cells ablated for PKAII (RII α) [11]. It should be noted that K_{act} in wild type sperm cells which mainly express PKAII (RII α - $C\alpha 2$) [45] is almost identical to the K_{act} in RII α ablated sperm cells. This may suggest that PKAI and PKAII display comparable K_{act} 's *in vivo* and hence contradicts the *in vitro* results demonstrated previously [38] and by us here. It should also be noted that PKAI although expressed at low levels may skew the observed K_{act} values, due to its sensitivity for cAMP. To what extent this has biological consequences as has been demonstrated for PKAI and PKAII in lymphoid cells [27;52;53], remains to be tested.

We then investigated the potency of the R subunits (RI α , RII α , RI β and RII β) and the protein kinase

inhibitor PKI α to inhibit $C\alpha 2$ phosphotransferase activity *in vitro*. Purified $C\alpha 2$ (30 nM) was mixed with a fixed concentration of Kemptide (250 μ M) in the presence of increasing concentrations of the various R subunits or PKI α . All the R subunits inhibited $C\alpha 2$ -dependent kinase activity by 50% at 15 nM and showed complete inhibition at a 1:1 molar ratio (Figure 5A and 5B). A fixed dose (28 nM) of $C\alpha 2$ was inhibited by PKI α in a dose-dependent manner with complete inhibition at stoichiometric concentrations of $C\alpha 2$ and PKI α (Figure 5C). The inhibitory effects of the various R subunits and PKI have previously been determined for $C\alpha 1$ [7;29] and indicate that the efficiency in inhibiting $C\alpha 1$ and $C\alpha 2$ is similar for all R subunits and PKI α . Using a Biacore technology we next investigated the dissociation equilibrium constants (K_D), association (k_{ass}) and dissociation (k_{diss}) rate constants for the various R subunits in association with either $C\alpha 1$ or $C\alpha 2$. We immobilized 300 RUs of myristylated $C\alpha 1$ (myr $C\alpha 1$, see Methods) and $C\alpha 2$ on separate flow cells of a CM5 Biacore sensor chip. Unmyristylated $C\alpha 1$ was used as reference (data not shown). In the presence of 1 mM ATP and 5 mM $MgCl_2$ the R subunits were simultaneously run over both C subunits on the sensor chip at a flow rate of 30 μ L/min. In the case of RI α and RI β they were run over the sensor chip at concentrations between 0.25 and 128 nM, and RII α and RII β between 0.5 to 256 nM (raw data not shown). Figure 6 (panel A) shows representative runs of RI α , RI β , RII α and RII β (64 nM each; panel B) on $C\alpha 2$ and myr $C\alpha 1$. The shape of the curves indicates that the relative on and off rates for RI α when associated with either $C\alpha 2$ or myr $C\alpha 1$ were highly similar. The relative k_{ass} values were slightly different, 1.6×10^6 and 1.9×10^6 $M^{-1}s^{-1}$ for RI α versus myr $C\alpha 1$ and $C\alpha 2$, respectively. The same was true for RI β . However, in this case, although the K_D value was almost identical to RI α , the association as well as the dissociation rate constants for RI β was 2 times faster. Finally, no differences could be observed for the interaction of RII α and RII β against myr $C\alpha 1$ versus $C\alpha 2$ (for rate and equilibrium constants see Table 1).

In order to investigate the binding behavior of PKI, GST-PKI α was immobilized on sensor chips as described previously [54], and various concentrations of $C\alpha 2$, $C\alpha 1$, myr $C\alpha 1$ and, for comparison, mouse $C\alpha 1$ were run at a flow rate of 30 μ L/min over the sensor chips. This revealed a K_D for all C subunits and PKI α at a range around 0,4 nM where the myr $C\alpha 1$ displayed a slightly faster association rate compared to $C\alpha 1$ (4.9×10^6 and 3.2×10^6 $M^{-1}s^{-1}$, respectively) with all the dissociation rates being similar (1.5×10^{-3} s^{-1}) (Table 2). SPR measurements with single concentrations demonstrated almost identical shapes of the curves (Figure 7),



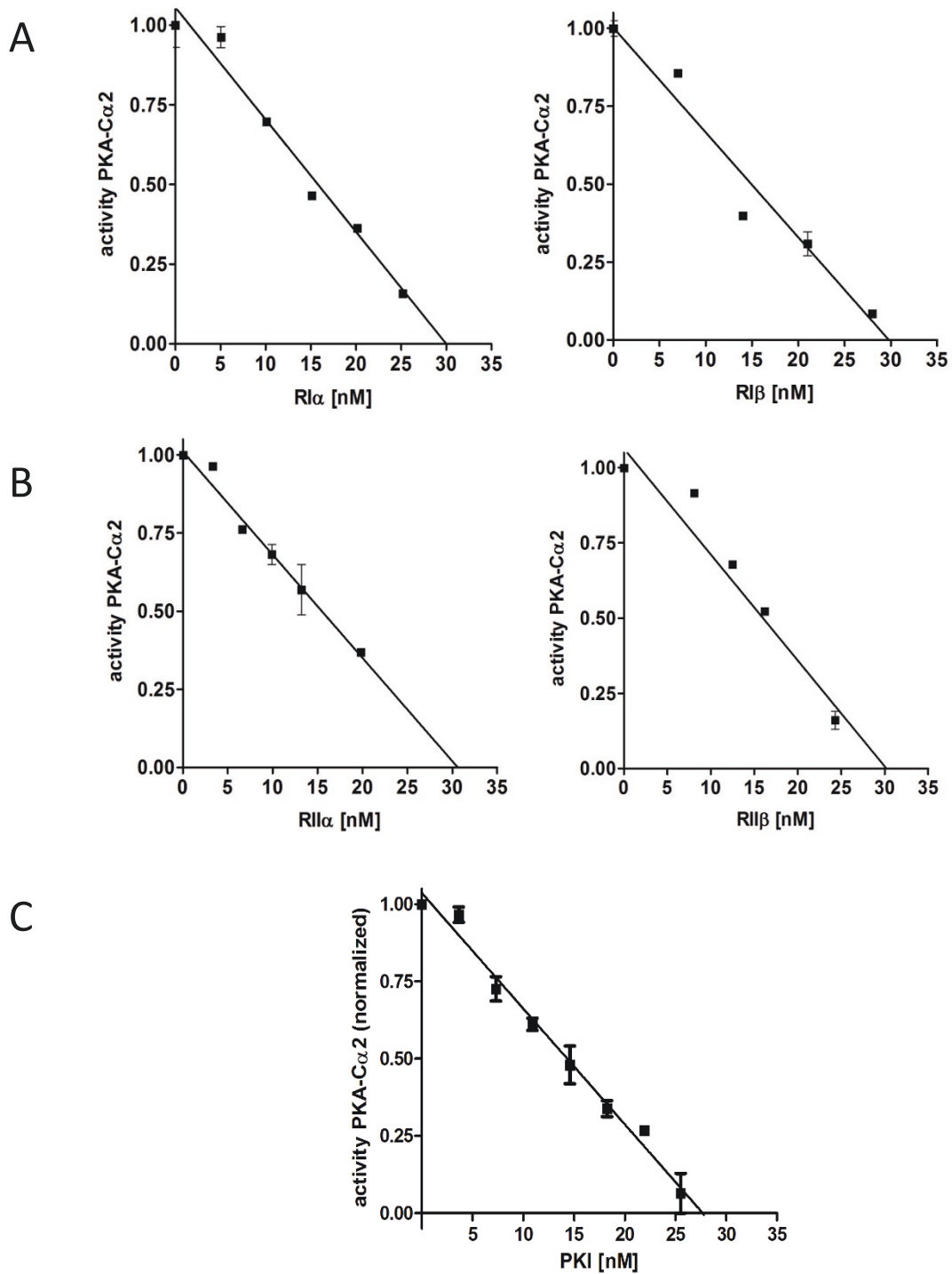
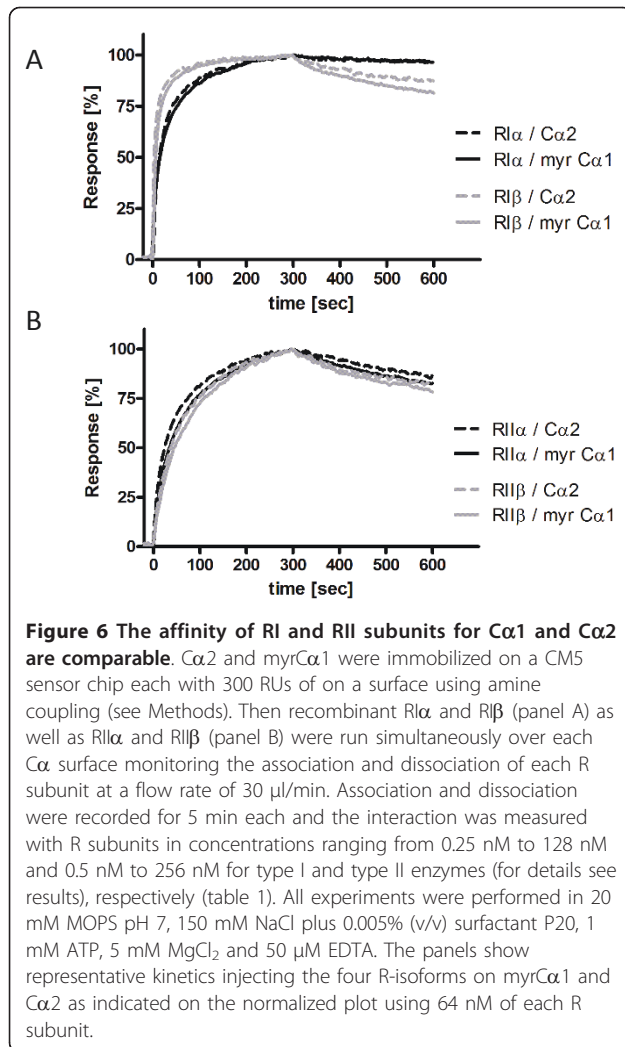


Figure 5 Phosphotransferase activity of C α 2 is inhibited by the R subunits and PKI in dose-dependent manner. Recombinant C α 2 (30 nM) mixed with Kemptide (250 μ M) and ATP (10 mM) was incubated in the presence of increasing concentrations (0 - 25 nM) of either RII α and RII β (panel A) and RII α and RII β (panel B). C α 2 is inhibited by all R subunits in a dose-dependent manner at equimolar concentrations of RII α , RII β , RII α and RII β . Panel C: The inhibitory effect of PKI α was verified by incubating recombinant C α 2 (28 nM) in the presence of Kemptide (250 μ M), ATP (10 mM) and increasing concentrations of recombinant PKI α (0-40 nM). Titration curves shown are normalized before linear regression (n = 2).



indicating comparable association and dissociation rates. In order to determine accurate association rate constants, different concentrations of the respective C subunits were applied (results not shown), leading again to the conclusion that the binding activities of $\text{Ca}2$ and $\text{Ca}1$ for PKI α are highly similar.

Table 1 Association and dissociation constants of RI and RII and $\text{Ca}1$ and $\text{Ca}2$

Analyt/Ligand (immobilized)	k_a [$\text{M}^{-1}\text{s}^{-1}$]	k_d [s]	K_D [nM]
hRI α /PKA- $\text{Ca}1$ myr	$1.6\text{E} + 6$	$200,0\text{E}-6$	0,13
hRI α /PKA- $\text{Ca}2$	$1.9\text{E} + 6$	$214,0\text{E}-6$	0,11
hRI β /PKA- $\text{Ca}1$ myr	$3.5\text{E} + 6$	$501,0\text{E}-6$	0,15
hRI β /PKA- $\text{Ca}2$	$4.4\text{E} + 6$	$482,0\text{E}-6$	0,11
hRII α /PKA- $\text{Ca}1$ myr	$1.0\text{E} + 6$	$469,0\text{E}-6$	0,48
hRII α /PKA- $\text{Ca}2$	$1.2\text{E} + 6$	$433,0\text{E}-6$	0,35
hRII β /PKA- $\text{Ca}1$ myr	$0.5\text{E} + 6$	$797,0\text{E}-6$	1,5
hRII β /PKA- $\text{Ca}2$	$0.9\text{E} + 6$	$793,0\text{E}-6$	0,9

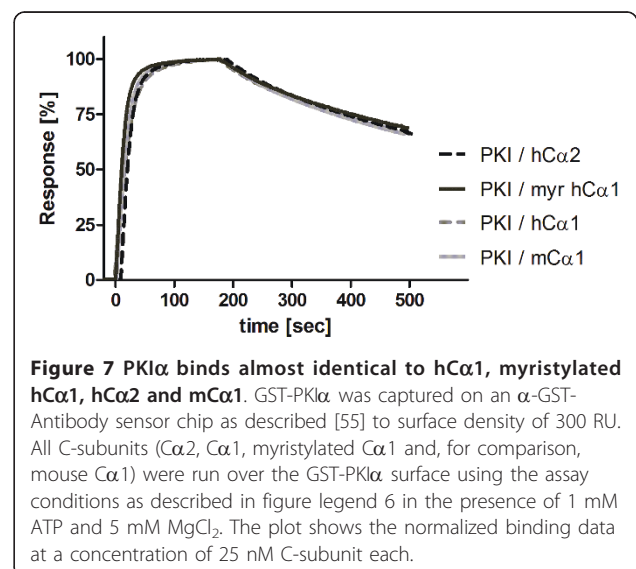
Table 2 Association and dissociation constants of GST-PKI α for $\text{Ca}1$ and $\text{Ca}2$

Analyt/Ligand (immobilized)	K_a [$\text{M}^{-1}\text{s}^{-1}$]	K_d [S]	K_d [nM]
GST-PKI α /PKA- $\text{Ca}1$	4.9×10^5	1.5×10^{-3}	0.5
GST-PKI α /PKA- $\text{Ca}2$	3.2×10^5	1.5×10^{-3}	0.7

Discussion

At the protein level $\text{Ca}1$ and $\text{Ca}2$ are 97% homologous and only differ at the N-terminal end. Based on this we investigated to what extent differences at the N-terminus may influence splice variant-specific activities that may have biological importance. We found that $\text{Ca}2$ expressed in bacteria was not captured by inclusion bodies as was the case with $\text{Ca}1$. Moreover, the specific activity of $\text{Ca}2$ was lower compared to $\text{Ca}1$. Apart from these differences we observed that $\text{Ca}2$ was highly similar to $\text{Ca}1$ in all parameters measured. This included association of $\text{Ca}2$ with RI and RII to form cAMP-sensitive holoenzymes both *in vivo* and *in vitro*. Furthermore, K_m values of $\text{Ca}2$ for Kemptide and ATP were comparable to those determined for $\text{Ca}1$. This was also the case for the ability of RI, RII and PKI to inhibit $\text{Ca}2$ enzyme activity *in vitro* (data not shown). Finally, K_D values as measured by SPR were shown to be comparable between $\text{Ca}1$ and $\text{Ca}2$ towards the RI and RII subunits as well as PKI.

Several reports imply that N-terminal modifications of $\text{Ca}1$ introduce specific features that may have biological consequences. To this end it has been suggested that phosphorylation of Ser10 in $\text{Ca}1$ introduces an electrostatic force which may help the C subunit to remain soluble even when myristylated [55;56]. Moreover, two reports have demonstrated that the N-terminal myristyl moiety of $\text{Ca}1$ is embedded in a hydrophobic pocket



encompassed in the large lobe [57;58]. Mutation of Gly1 to Ala rendering the $C\alpha 1$ non-myristylated, demonstrated that myristylation was non-essential for conformation and enzyme activation, and was not required for $C\alpha 1$ interaction with other proteins including various substrates and the R subunits [21,59]. The fact that $C\alpha 2$ is not myristylated and displays comparable activities with myristylated $C\alpha 1$ suggests that myristylation is not essential for catalytic activity, holoenzyme formation and inhibition by PKI. This is further supported in that deletion of the entire $C\alpha 1$ N-terminus did not severely interfere with catalytic activity and inhibitor binding despite that deletion caused thermo instability [25]. This also suggests that the amino acids 2 (Asn) and 10 (Ser) of $C\alpha 1$ are not essential for activity a suggestion which is supported by our results on $C\alpha 2$.

$C\alpha 1$ and $C\beta 1$ which are 100% identical at the N-terminus, but only 91% identical in the sequence encode by exon 2 through 10, have different apparent sizes (40 and 41 kDa) and possess distinct biochemical properties both *in vitro* and *in vivo* [60; 31]. These differences include differential K_m values for certain peptide substrates and that $C\alpha 1$ but not $C\beta 1$ is inhibited by substrate concentrations above 100 μ M. In addition, they display distinct IC_{50} values for PKI and $R_{II}\alpha$. Taken together with our results this may suggest that the amino acid sequence encoded by exon 2 through 10 and not exon 1 influence C subunit activities such as holoenzyme formation, enzyme activity and inhibition by R and PKI. This may further imply that $C\alpha 1$ and $C\beta 1$ have distinct roles in regulating cellular processes. This was recently shown in T cells which express $C\alpha 1$, $C\beta 1$ and $C\beta 2$. In that study $C\alpha 1$, but none of the $C\beta$ forms, mediated the inhibitory effect of cAMP on immune cell reactivity *in vivo* [17;61]. In light of these observations it is also of interest to note that $C\alpha 2$ but not $C\alpha 1$ is required for sperm cell forward velocity and male fertility, despite 100% identity at the amino acid sequence encoded by exon 2 through 10 [20;62]. However, since $C\alpha 2$ is the sole C subunit in sperm cells [9;10;63], the difference observed may only be ascribed to tissue-specific expression and not sequence-specific differences.

In contrast to $C\alpha 1$ it is expected that the hydrophobic pocket in which the myristyl group is submersed in $C\alpha 1$, is constitutively empty and exposed to the surroundings at all time in non-myristylated $C\alpha 2$. It has been speculated whether exposure of the hydrophobic pocket would introduce more lipophilic properties to the $C\alpha 2$ subunit [64]. Support for such a hypothesis is found in a by a study demonstrating that binding of $C\alpha 1$ to R_{II} induced a unique conformation that is associated with exposure of the hydrophobic pocket to the surroundings due to increase in N-terminal

flexibility of the N-myristate away from the large lobe. This renders $C\alpha 1$ more hydrophobic and promotes membrane association of the PKA II holoenzyme only [64]. Therefore it may be suggested that exposure of the hydrophobic pocket serves features such as isoform specific features and subcellular localization of the C subunit. To this end it is interesting to note that $C\alpha 2$ is associated with the sperm tail in the presence of detergent treatment with 1% Triton X-100 .and, after a challenge with 2 mM cAMP [10]. This may be indicative of a direct association of $C\alpha 2$ with subcellular structures. To what extent such attachment involves the hydrophobic pocket remains unknown. In other cells and tissues, C subunits targeted to subcellular structures independent of the R subunit and traditional A-kinase anchoring proteins have been demonstrated. To day a number of C subunit binding proteins have been identified. These include PKI, A-kinase interacting protein 1 (AKIP1), homologous to AKAP95 (HA95), inhibitor of NFkappaB kinase ($I\kappa$ B), Caveolin-1 and p75 neutrophine receptor (p75NTR) [65-69]. To what extent $C\alpha 2$ is targeted to the sperm cell midpiece through a C interaction protein and if specificity of binding is retained in the hyper variable N-terminal end remains to be shown. However, it should be noted that deamination of the Asn2 moiety in $C\alpha 1$ helps fine-tuning enzyme distribution within the cell *in vivo* [70]. Moreover, p75NTR was shown to specifically bind to the $C\beta$ splice variant $C\beta 4ab$ [69], which is encoded with unique N-terminal domain that may not undergo the same posttranslational modifications as $C\alpha 1$ [15;69]. Together this may imply that the N-terminal end may be important for targeting and specificity of subcellular localization of the various C subunits.

Conclusion

Our study demonstrates that N-terminal sequence encoded by alternative use of exons upstream of exon 2 in the PRKACA gene does not influence C subunit activities such as holoenzyme formation, cAMP sensitivity, enzyme activity as well as inhibition by RI, RII and PKI. Based on several studies it may be suggested that the N-terminus is involved in other C subunit features such as subcellular localization.

Abbreviations

PKA: Protein kinase A; C: Catalytic subunit; C α : Alpha-form of C; C $\alpha 2$: Sperm-specific C subunit.

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Author details

¹Department of Biochemistry, University of Kassel, Heinrich-Plett-Str, 40, 34132 Kassel, Germany. ²Department of Nutrition, Institute of Basic Medical Sciences, University of Oslo, Pb 1046 Blindern, 0316 Oslo Norway.

³Department of Biochemistry, Institute of Basic Medical Sciences, University of Oslo, Pb 1046 Blindern, 0316 Oslo Norway.

Authors' contributions

MMV has written the manuscript and has designed and contributed to the experiments in figures 3 through 7 and table 1 and 2. HMZ has contributed to the BIACORE experiments. EM has performed the experiments in figures 1 and 2. HVB has made recombinant proteins for Ca1 and Ca2 and contributed to the experiments in figure 3. FWH and BSS have conceived the ideas to the manuscript and contributed to the writing. All authors read and approved the final manuscript.

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