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# Inactive forms of the catalytic subunit of protein kinase A are expressed in the brain of higher primates

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It is well documented that the β-gene of the catalytic (C) subunit of protein kinase A encodes a number of splice variants. These splice variants are equipped with a variable N-terminal end encoded by alternative use of several exons located 5′ to exon 2 in the human, bovine and mouse Cβ gene. In the present study, we demonstrate the expression of six novel human Cβ mRNAs that lack 99 bp due to loss of exon 4. The novel splice variants, designated CβΔ4, were identified in low amounts at the mRNA level in NTera2-N cells. We developed a method to detect CβΔ4 mRNAs in various cells and demonstrated that these variants were expressed in human and Rhesus monkey brain. Transient expression and characterization of the CβΔ4 variants demonstrated that they are catalytically inactive both *in vitro* against typical protein kinase A substrates such as kemptide and histone, and *in vivo* against the cAMP-responsive element binding protein. Furthermore, co-expression of CβΔ4 with the regulatory subunit (R) followed by kinase activity assay with increasing concentrations of cAMP and immunoprecipitation with extensive washes with cAMP (1 mM) and immunoblotting demonstrated that the CβΔ4 variants associate with both RI and RII in a cAMP-independent fashion. Expression of inactive C subunits which associate irreversibly with R may imply that CβΔ4 can modulate local cAMP effects in the brain by permanent association with R subunits even at saturating concentrations of cAMP.

Differential exon use is a hallmark of alternative splicing, a prevalent mechanism for generating protein isoform diversity. There are two principal genes encoding the catalytic (C) subunit of cAMP-dependent protein kinase A, termed Cα and Cβ [1]. Both the Cα and Cβ genes transcribe several splice variants, which are termed Cα1, CαS, Cβ1, Cβ2, Cβ3, Cβ3b, Cβ3ab, Cβ3abc, Cβ4, Cβ4b, Cβ4ab and Cβ4abc [2–6]. All the known C subunit splice variants are encoded with variable N-terminal ends due to alternative splicing of exon 1 and differential splicing of exons a, b and c. Interestingly, the N-terminus of Cα1 and Cβ1 are more homologous to each other than to any of their splice variants. In the case of Cα1, three sites may undergo co- and post-translational modifications. At the very N-terminus, Cα1 is encoded with a Gly that is myristoylated *in vivo* [7]. Moreover, C-terminal to the Gly an Asn is encoded that is partly deamidated *in vivo*, leading to Cα1-Asp$^2$ and Cα1-iso(β)Asp$^2$ [8]. Finally, a third modification is identified as a protein kinase A

**Abbreviations**
C, catalytic subunit; CRE, cAMP-regulated element; NT2, NTera-2; PBL, peripheral blood leukocyte; PKA, protein kinase A; R, regulatory subunit; TBST, NaCl/Tris with 0.1% Tween-20.

(PKA)-autophosphorylation site at $Ser^{10}$ [9–11]. Based on the fact that Cα1 and Cβ1 have identical amino acid sequences where the modification takes place, it is expected that Cβ1 is modified in the same way as Cα1. Despite this, Cα1 has a three- to five-fold lower $K_m$ for certain peptide substrates than does the Cβ1, in addition to a three-fold lower $IC_{50}$ for inhibition by PKI and regulatory subunit (R) IIα [12], implying that other domains different from the N-terminus may influence C subunit features.

None of the other known C splice variants are encoded with the same N-terminus as Cα1 and Cβ1 and it is not expected that they undergo the same type of modifications. Thus, they may harbor different features than those of Cα1 and Cβ1. This has been demonstrated for the Cα splice variants in that CαS, but not Cα1, regulates sperm motility [13,14]. Moreover, the N-terminal end has been suggested to play a role in regulating enzyme activity and protein stability, as well as subcellular targeting of the C. The latter has recently been demonstrated in that the N-terminal residues 1–39 are required for localization of A-kinase interaction protein in the nucleus [15]. Despite these reports, specific functions associated with the various N-terminal ends of the PKA C subunits are elusive.

Alternative splicing of the Cα and Cβ genes appears to be tissue specific in that Cα1 and Cβ1 are ubiquitously expressed, whereas CαS is only expressed in sperm cells [2,3,16]. Cβ2 appears to be expressed mainly in lymphoid tissues, whereas the Cβ3 and Cβ4 and their abc variants are expressed primarily in the central nervous system [5,6,17,18].

In the present study, we show that human NTera2-N (NT2-N) cells, which are differentiated by retinoic acid for 4 weeks from NT2 cells to NT2-N cells with characteristics of post-mitotic neurons of the central nervous system [19], express six novel mRNA species of the PKA Cβ gene; these variants lack exon 4. The Cβ forms lacking exon 4 were detected in nerve cells of human and Rhesus monkey. The novel splice variants were shown to be catalytically inactive because they did not phosphorylate PKA substrates either *in vitro* or *in vivo*. Finally, we established that the Cβ variants lacking the exon 4 were able to interact with the PKA R subunits in a cAMP-insensitive manner.

## Results

We have previously demonstrated that a number of different Cβ splice variants are induced in NT2 cells during retinoic acid-dependent differentiation for 4 weeks into NT2-N cells [6]. A search in the expressed sequence tag database revealed the sequence of Cβ3ab lacking the 99 bases of exon 4 (accession number AK091420). To verify the existence of Cβ splice variants lacking exon 4, we performed RT-PCR using different primers pairs (Fig. 1A). To determine whether exon 4 skipping occurs both for Cα and Cβ, we applied two primer pairs spanning exon 4, recognizing all Cα (Cα common primer pair; upper and lower primers annealing in exons 3 and 6, respectively) or Cβ (Cβ common primer pair; upper and lower primers annealing in exons 3 and 9, respectively) isoforms. Furthermore, we used Cβ splice variant specific upper primers, as described previously [6], but in combination with lower primers corresponding to Cβ-specific sequences in exons 8 or 9 to investigate whether exon 4 exclusion occurs for all known Cβ splice variants. Figure 1B shows that the PCR reaction using the Cβ common primer pair yielded two visible bands (lane 2), whereas the PCR reaction using the Cα primer pair produced only one band (lane 1), suggesting that the exon 4 exclusion is Cβ specific. Figure 1C demonstrates that the Cβ splice variant specific primer pairs all yielded at least two detectable bands. The PCR products were cloned, sequenced and the sequences aligned with the published PKA Cβ sequences, revealing six novel PKA Cβ splice variants lacking the 99 bp encoded by exon 4. They were designated Cβ1Δ4, Cβ2Δ4, Cβ3Δ4, Cβ3abΔ4, Cβ3abcΔ4 and Cβ4abΔ4. To establish that the CβΔ4 variants existed as full-length transcripts, we performed RT-PCRs with the Cβ specific upper primers (Table 1) combined with a lower primer in exon 10 (results not shown). The nucleotide sequence of Cβ3Δ4 was translated to the amino acid sequence and compared with the full-length Cβ3 amino acid sequence (Fig. 2). This demonstrated that Cβ3Δ4 lacks the 33 amino acids encoded by exon 4.

The fact that the CβΔ4 variants were expressed in NT2-N cells prompted us to investigate whether these variants are found in other human Cβ-expressing tissues, such as brain [20] and immune cells [5,18]. Human brain and peripheral blood leukocyte (PBL) cDNA was PCR amplified using the Cβ common primer pair (Table 1, primers V and VII) and NT2-N cDNA was included as a control. This revealed that a shorter Cβ fragment co-migrating with the shorter band seen in NT2-N cells is present in brain, but not in PBL (Fig. 3A, lanes 2 and 3). To examine whether the CβΔ4 variants were expressed in different parts of the brain as well as in fetal brain, PCR was carried out using the Cβ common primer pair on cDNA from hippocampus, amygdala and cerebral cortex of human adult brain, and on cDNA from human fetal brain. Cβ was barely detectable in fetal brain (Fig. 3B, lane 1)

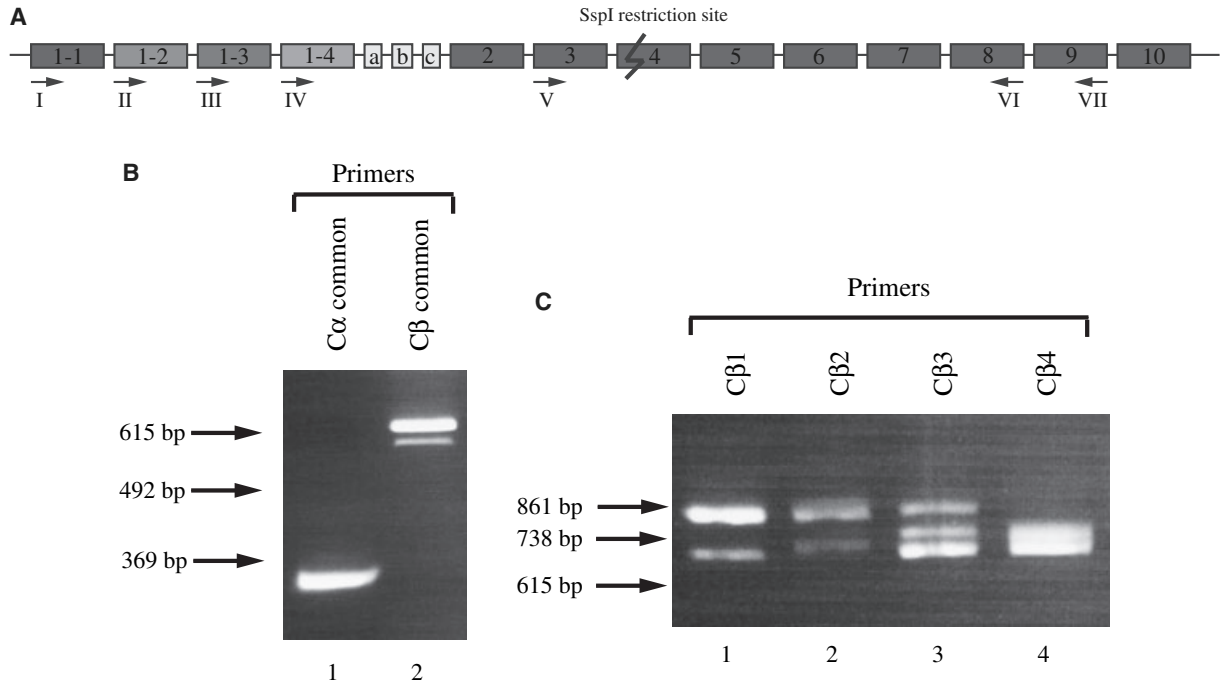


**Fig. 1.** Exon 4 exclusion occurs for Cβ, but not for Cα. Complementary DNA was generated from NT2-N cell total RNA and used as template in PCR reactions with primers recognizing all Cβ and Cα variants (Cβ common and Cα common, respectively) and splice variant specific primers amplifying Cβ1, Cβ2 and the various Cβ3 and Cβ4 variants. PCR products were separated on a 1% agarose gel and visualized by ethidium bromide staining. Arrows indicate migration of the DNA standards. Negative control reactions, in which cDNA was not added yielded no detectable PCR fragments (data not shown). (A) A schematic representation of the human PKA Cβ gene structure. Location of the Cβ primers used in RT-PCR is indicated and refers to primers listed in Table 1. The *Ssp*I restriction site in exon 4 is also shown. (B) The common primers for Cβ yielded products of 630 and 531 bp (lane 2) whereas the common primers for Cα resulted in one product of 343 bp (lane 1). (C) Cβ1 and Cβ2 primers yielded products of 838 and 739 bp, and 808 and 709 bp, respectively (lanes 1 and 2). Cβ3 and Cβ4 variant primer pairs resulted in several bands with lengths between 888 and 732 bp (lanes 3 and 4).

**Table 1.** Primers used for PCR amplification (all Sigma-Genosys Ltd, noncommercial; roman numbers in parenthesis refer to primers indicated in Fig. 1A).

| Primer pair | Upper primer (5′- to 3′) | Lower primer (5′- to 3′) |
|---|---|---|
| Cα common, human | CGGGAACCACTATGCC | GTAGCCCTGCTGGTCAATGA |
| Cβ common, human | ACACAAAGCCACTGAA (V) | TTCCGTAGAAGGTCCTTGAG (VII) |
| Cβ1, human | CCCTTCTTGCCATCG (I) | TTCCGTAGAAGGTCCTTGAG (VII) |
| Cβ2, human | GCCGGTTATTTCATAGACAC (II) | CCTAATGCCCACCAATCCA (VI) |
| Cβ3, human | AAGACGTTTAGGTGCAAT (III) | TTCCGTAGAAGGTCCTTGAG (VII) |
| Cβ4, human | CCCTTTGCTGTTGGAT (IV) | TTCCGTAGAAGGTCCTTGAG (VII) |
| Cβ common, Rhesus monkey | TGCCATGAAGATCTTAGA | CTAATCTATGAAATGGCAG |
| Cβ common, mouse | TGAGCAGTACTACGCCATGA | TCCACCGCCTTATTGTAACC |

whereas a higher level of expression was apparent in all adult brain sections examined (Fig. 3B, lanes 2–5).

To diminish the possibility that PBL express CβΔ4 variants at levels below the detection limit of normal PCR, we developed a more sensitive method for CβΔ4 mRNA detection. In this method, the Cβ variants were amplified by PCR using the Cβ common primer pair as described above. To increase the probability of detecting any CβΔ4 variants, the amplified DNA was treated with the restriction enzyme *Ssp*I, which has a unique restriction site in the human Cβ exon 4 sequence. *Ssp*I activity cleaves the full-length fragments containing exon 4, but leaves the CβΔ4 fragments intact. When the *Ssp*I-digested reaction is re-amplified by PCR, only the remaining CβΔ4 variants will be amplified. Figure 4 shows the results of experiments with cDNA from NT2-N cells, human adult brain and PBL after applying this method. A PCR product

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1   MGLLKEFLAKAKEDFLKKWENPTQNNAGLEDFERKKTLGTGSFGRVMLVKHKATEQYYAM 60
    MGLLKEFLAKAKEDFLKKWENPTQNNAGLEDFERKKTLGTGSFGRVMLVKHKATEQYYAM
1   MGLLKEFLAKAKEDFLKKWENPTQNNAGLEDFERKKTLGTGSFGRVMLVKHKATEQYYAM 60

61  KILDKQKVVKLKQIEHTLNEKRILQAVNFPFLVRLEYAFKDNSNLYMVMEYVPGGEMFSH 120
    KILDKQK                                 DNSNLYMVMEYVPGGEMFSH
61  KILDKQK---------------------------------DNSNLYMVMEYVPGGEMFSH 87

121 LRRIGRFSEPHARFYAAQIVLTFEYLHSLDLIYRDLKPENLLIDHQGYIQVTDFGFAKRV 180
    LRRIGRFSEPHARFYAAQIVLTFEYLHSLDLIYRDLKPENLLIDHQGYIQVTDFGFAKRV
88  LRRIGRFSEPHARFYAAQIVLTFEYLHSLDLIYRDLKPENLLIDHQGYIQVTDFGFAKRV 147

181 KGRTWTLCGTPEYLAPEIILSKGYNKAVDWWALGVLIYEMAAGYPPFFADQPIQIYEKIV 240
    KGRTWTLCGTPEYLAPEIILSKGYNKAVDWWALGVLIYEMAAGYPPFFADQPIQIYEKIV
148 KGRTWTLCGTPEYLAPEIILSKGYNKAVDWWALGVLIYEMAAGYPPFFADQPIQIYEKIV 207

241 SGKVRFPSHFSSDLKDLLRNLLQVDLTKRFGNLKNGVSDIKTHKWFATTDWIAIYQRKVE 300
    SGKVRFPSHFSSDLKDLLRNLLQVDLTKRFGNLKNGVSDIKTHKWFATTDWIAIYQRKVE
208 SGKVRFPSHFSSDLKDLLRNLLQVDLTKRFGNLKNGVSDIKTHKWFATTDWIAIYQRKVE 267

301 APFIPKFRGSGDTSNFDDYEEEDIRVSITEKCAKEFGEF 339
    APFIPKFRGSGDTSNFDDYEEEDIRVSITEKCAKEFGEF
268 APFIPKFRGSGDTSNFDDYEEEDIRVSITEKCAKEFGEF 306
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**Fig. 2.** Comparison of Cβ3 and Cβ3Δ4 amino acid sequences. RT-PCR products using Cβ3-specific primers were cloned, sequenced and shown to contain both short and long nucleotide products. The DNA sequences of the short product was translated to amino acid sequence (lower line) and compared with the published PKA Cβ3 sequences (upper line). The shorter DNA shows 100% identity to Cβ3, but lacks the 33 amino acids encoded by exon 4 (bold).

corresponding to the CβΔ4 variants is observed in NT2-N cells and brain after *Ssp*I treatment (Fig. 4, lanes 4 and 8, respectively), but not in PBL (Fig. 4, lane 12). A weak upper band representing incomplete *Ssp*I digestion of the exon 4-containing fragments is present in lane 8. Negative control samples in which cDNA was omitted, with (+) and without (−) *Ssp*I treatment were also performed (lanes 1, 2, 5, 6, 9 and 10). Taken together, these results suggest that CβΔ4 variants are not expressed in human PBL.

Next, we searched for these splice variants in the brain of other species. Rhesus monkey brain and mouse brain cDNAs were PCR amplified using the human and mouse Cβ common primers (Table 1), respectively. The resulting DNA fragments were treated or not treated with *Ssp*I (monkey) or *Pst*I (mouse) before being re-amplified with the same primers. This yielded two DNA bands of the expected sizes from monkey brain cDNA, but not for mouse cDNA (data not shown). To verify that the lower band represents PKA Cβ, the PCR products were cloned and sequenced. Because no Rhesus monkey PKA C subunit sequences have been published, we compared this sequence with the human Cβ sequence. This revealed two nucleotide differences between the two species (Fig. 5) and the 99 bases of exon 4 were missing. The variation in nucleotides was not revealed at the amino acid level (see Supplementary Material, Fig. S1). In conclusion, these results demonstrate that CβΔ4 variants are expressed in Rhesus monkey brain but probably not in mouse brain.

As depicted in Fig. 6A, exon 4 encodes an α-helix in the outer border of the catalytic domain in Cα1 (yellow line), suggesting that deletion may notably affect the catalytic activity of the CβΔ4 variants. Expression plasmids for native Cβ1, Cβ1Δ4, Cβ3ab and Cβ3abΔ4 were made and transfected into 293T cells. The cell lysates were monitored for *in vitro* PKA-specific phosphorylation activity using the PKA-specific substrate kemptide and the endogenous PKA substrate histone H1. All plasmids expressed immunoreactive C subunits above mock levels (Fig. 6B, upper panel). Figure 6B demonstrates that Cβ1Δ4 and Cβ3abΔ4 are catalytically inactive against kemptide (middle panel) and histone (lower panel) compared to the catalytic activity monitored in cells transfected with Cβ1 and Cβ3ab. Furthermore, Cβ1, Cβ1Δ4, Cβ3ab and Cβ3abΔ4 were tested for the ability to induce a cAMP-regulated element (CRE)-regulated promoter in the *in vivo* luciferase reporter assay. 293T cells were co-transfected with a CRE-luc reporter plasmid, a β-galactosidase control plasmid and each of the Cβ expression vectors.

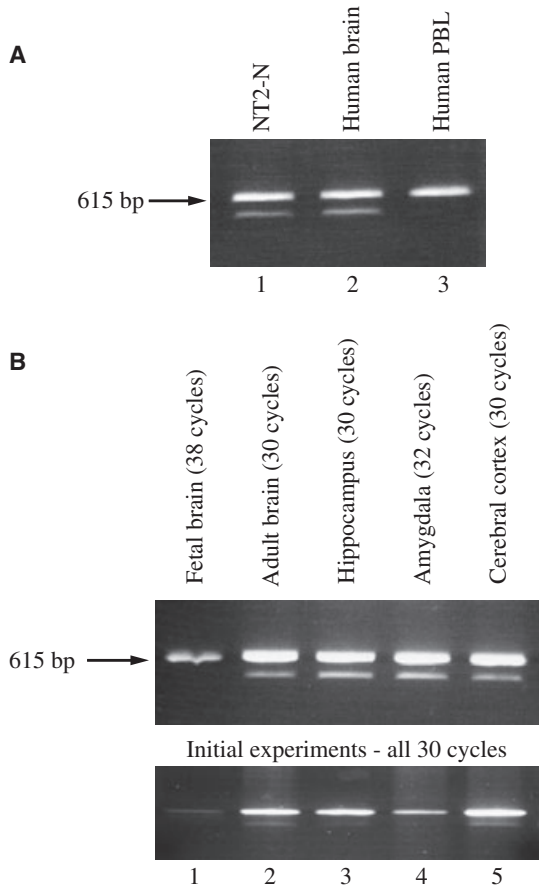


**Fig. 3.** Cβ splice variants lacking exon 4 are expressed in several compartments of the human brain. (A) Complementary DNA prepared from NT2-N cells, human brain and human peripheral blood leukocytes were used as templates in PCR reactions using the Cβ common primers (upper primer in exon 3 and lower primer in exon 9). PCR products were separated by 1% agarose gel electrophoresis and stained with ethidium bromide. PCR reactions yielded products of 630 and 531 bp for both the NT2-N and human brain cells (lanes 1 and 2) and a 630 bp product for human peripheral blood leukocytes (lane 3). Arrow indicates migration of the DNA standard. (B) PCR ready cDNA from human fetal brain, human adult brain, human adult hippocampus, amygdala and cerebral cortex were used as templates in PCR reactions with the Cβ common primers. A 630 bp product was detected in all reactions after 30 PCR cycles (lower panel). However, 38 PCR cycles were necessary to obtain a clear dense band representing Cβ in fetal brain (upper panel, lane 1). Thirty to 32 cycles was sufficient to produce a 531 bp product in human adult brain, hippocampus, amygdala and cerebral cortex (lanes 2–5, respectively). Arrow indicates migration of the DNA standard.

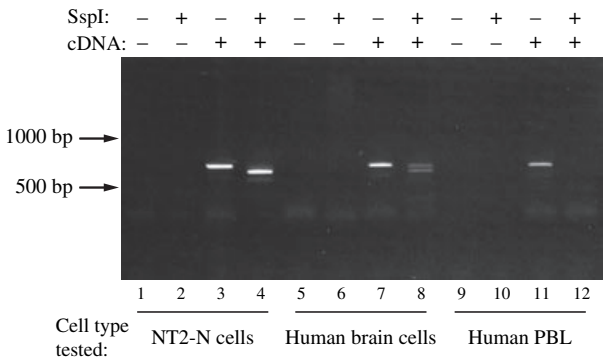


**Fig. 4.** CβΔ4 variants are expressed in human nerve cell tissue, but not in human peripheral blood leukocytes. Complementary DNA from NT2-N cells, human brain and peripheral blood leukocytes were PCR amplified using the Cβ common primers. DNA from the first PCR reaction was either left untreated (–) or treated (+) with *Ssp*I to digest exon 4-containing products and re-amplified in a second PCR reaction (see Experimental procedures) using the Cβ common primers. Parallel reactions without cDNA served as negative controls (lanes 1 and 2, 5 and 6, 9 and 10). In re-amplified reactions not treated with *Ssp*I, a 630 bp DNA fragment was detected for all cell types tested (lanes 3, 7 and 11). In reactions treated with *Ssp*I, a 531 bp fragment was identified for NT2-N and human brain cells (lanes 4 and 8), but not for PBL (lane 12). A weak 630 bp band detected in lane 8 represents incomplete digestion of exon 4 containing fragments in this reaction. Arrows indicate migration of the DNA standard.

All Cβ variants were expressed, as determined by immunoblot analysis (Fig. 6C, upper panel), and none of the CβΔ4 variants were able to induce luciferase activity above background (mock) level, whereas the normal Cβ variants induced activity far above mock levels (Fig. 6C, lower panel). Taken together, the results in Fig. 6B and C suggest that the PKA CβΔ4 variants are catalytically inactive.

In living cells, cAMP levels regulate the association of the R and C subunits to form PKA holoenzymes [21]. To explore whether the CβΔ4 containing holoenzymes display altered cAMP sensitivity, we co-expressed RIα with either Cβ1 or Cβ1Δ4 in 293T cells followed by measurements of PKA-specific phosphotransferase activity against kemptide at increasing concentrations of cAMP. To correlate cAMP sensitivity between PKA holoenzymes containing Cβ1 or Cβ1Δ4, we ensured approximately equal expression levels of RIα, Cβ1 and Cβ1Δ4 in each experiment based on immunoblot analysis. This demonstrated that RIα was expressed at equal levels and that Cβ1 was expressed at a comparable level relative to Cβ1Δ4 (Fig. 7A, inserts). When monitoring C subunit activity, we observed an expected dose-dependent increase in catalytic activity for Cβ1 by cAMP which was more than four-fold above the maximum levels of endogenous C subunit activity monitored in mock-transfected cells at the same cAMP concentrations (Fig. 7A). It should be noted that C subunit activity in Cβ1 transfected cells was comparable to mock activity at low cAMP

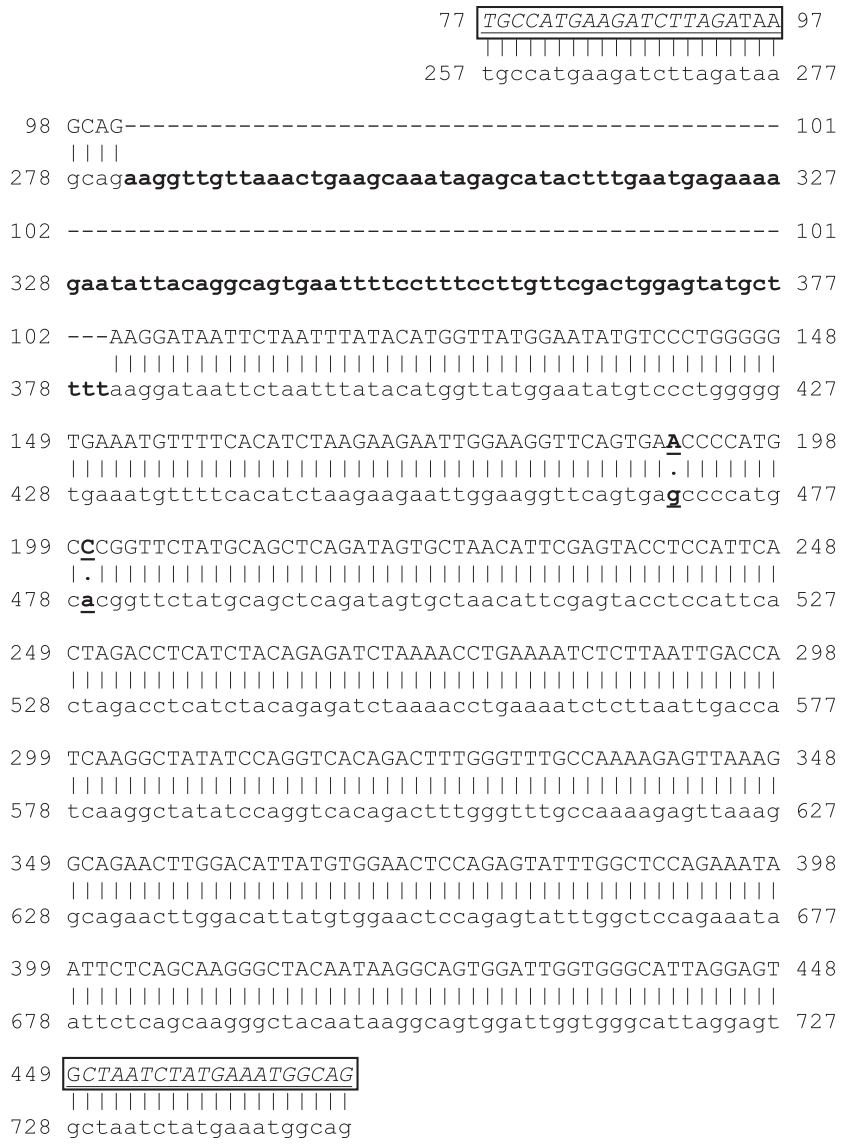


**Fig. 5.** CβΔ4 variants are expressed in Rhesus monkey brain. Complementary DNA from Rhesus monkey brain was PCR amplified using the Cβ common primers. Separation of PCR products by 1% agarose gel electrophoresis and visualization by ethidium bromide staining revealed two bands of the expected sizes. Both bands were cloned and sequenced. The DNA sequence of the short PCR product from monkey brain was compared with the human Cβ sequence (monkey, capital letters and human, lower case). Note that the 99 bp corresponding to exon 4 is lacking in monkey Cβ sequence. The human exon 4 sequence is shown in bold. Primers used in PCR reactions are boxed and in italic. Two nucleotides in the monkey sequence that are different from the human sequence (A – g and C – a) are underlined and shown in bold.

concentrations (0.005 μM) implying that all transfected Cβ1 was in the holoenzyme form. When RIα was co-transfected with Cβ1Δ4, we did not detect an altered maximum kinase activity compared to mock-transfected cells even at the highest cAMP concentrations (15 μM) and despite that Cβ1Δ4 appeared to be expressed at comparable levels to Cβ1 (Fig. 7A, upper insert). This confirms our findings of an inactive CβΔ4 and also indicates a complete and continuous association of RIα and Cβ1Δ4 because neither cAMP sensitivity nor maximum activity of the endogenous PKA holoenzymes appeared to be affected by the relative high levels of transfected PKA subunits. The presence of a cAMP-insensitive R and CβΔ4 interaction is substantiated by the fact that this was evident even at high concentrations of cAMP (15 μM). To further investigate the latter observation, 293T cells were transfected with RIα or RIIα in conjunction with one of the following C subunits: Cβ1, Cβ1Δ4, Cβ3ab or Cβ3abΔ4. Twenty to twenty-four hours post-transfection, cell lysates were immunoprecipitated with either anti-RIα or anti-RIIα sera, depending on the transfected R subunit. Immunoblots using anti-C serum showed that both the exon 4-containing and the exon 4-lacking Cβ variants were precipitated by anti-R serum (Fig. 7B, lanes 1 and 5), implying that both RIα and RIIα associates with the novel CβΔ4 subunits *in vivo*. To test whether the interactions are cAMP sensitive, the immunoprecipitates were incubated in the absence (–) and presence (+) of 1 mM cAMP, and pellet and

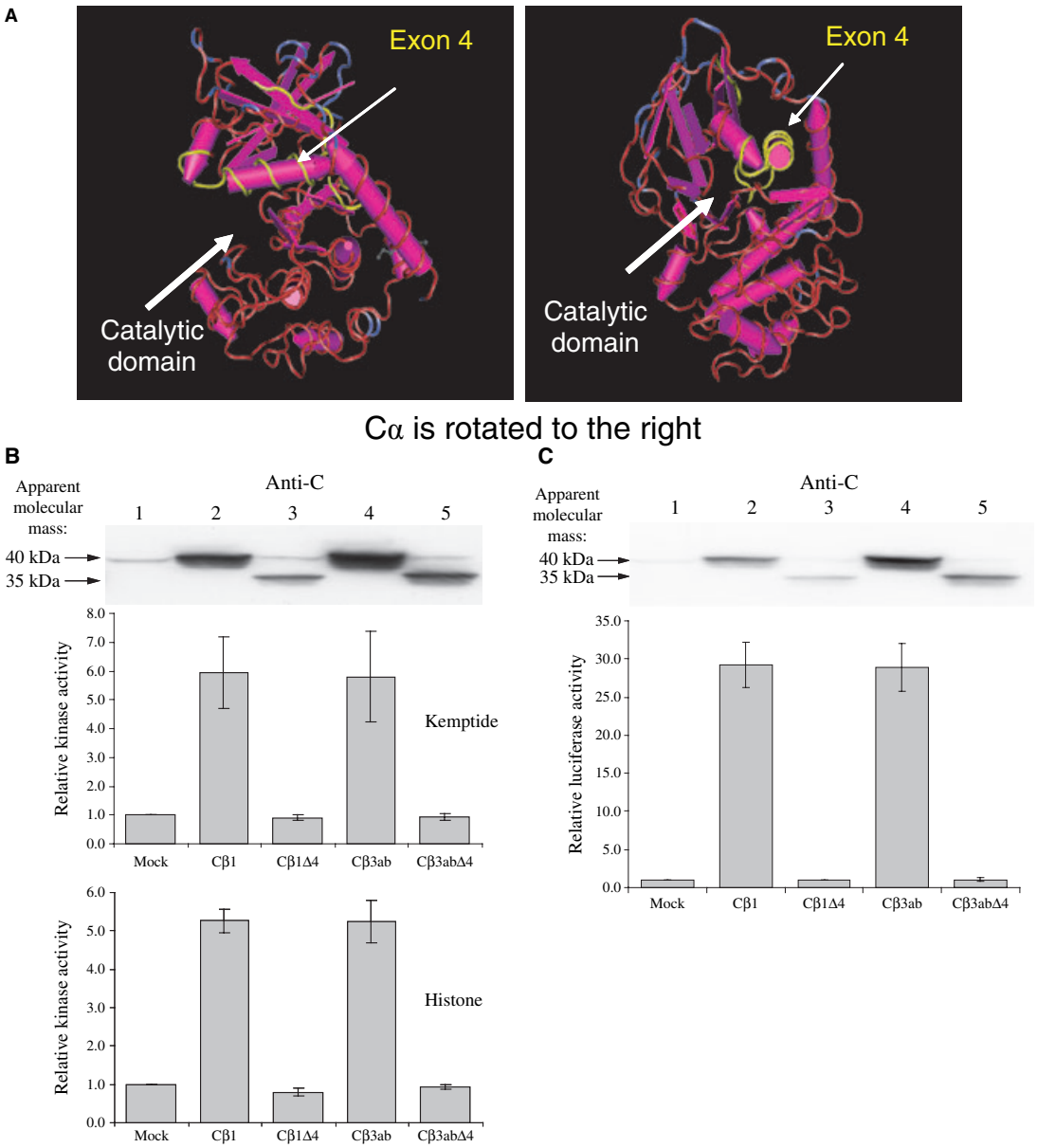


**Fig. 6.** CβΔ4 variants are catalytically inactive. (A) Three dimensional structure of Cα1. The exon 4 encoded sequence is outlined in yellow and indicated by a thin arrow. The thick arrow indicates the catalytic cleft. Adapted from [27], using the CN3D software, version 4.1 (National Centre for Biotechnology Information, Bethesda, MD, USA). (B) Expression and catalytic activities of Cβ1, Cβ1Δ4, Cβ3ab and Cβ3abΔ4. Cell extracts of 239T cells, either mock transfected or transfected with expression vectors for Cβ1, Cβ1Δ4, Cβ3ab and Cβ3abΔ4, were analysed by immunoblotting using a pan-C antibody (upper panel). Immunoreactive PKA C subunits of approximately 40 kDa are clearly recognized in Cβ1 and Cβ3ab transfected cells (lanes 2 and 4) whereas a 35 kDa band is recognized in the CβΔ4 transfected cells (lanes 3 and 5). Apparent molecular masses are indicated by arrows. The same cell extracts were monitored for PKA-specific kinase activity using γ-[$^{32}$P]ATP and the PKA substrates kemptide (middle panel) and histone (lower panel). Relative kinase activities were compared with PKA activity in mock transfected cells and are presented as the mean ± SEM from three representative experiments. (C) 239T cells were co-transfected with a CRE-luciferase reporter plasmid, a β-galactosidase control plasmid and one of the following expression vectors: Cβ1, Cβ1Δ4, Cβ3ab and Cβ3abΔ4. Mock samples were transfected with the CRE-luciferase reporter plasmid and β-galactosidase control plasmid only. Cell lysates were analyzed for C subunit expression levels by immunoblotting using a pan-C antibody (upper panel). A 40 kDa immunoreactive band is clearly recognized in Cβ1 and Cβ3ab transfected cells (lanes 2 and 4). A 35 kDa immunoreactive band is detected in lanes 3 and 5. Arrows indicate apparent molecular masses. The cell lysates were monitored for luciferase activity (lower panel). The relative levels of luciferase activity were compared with the activity in mock transfected cells and are presented as the mean ± SEM from three representative experiments with luciferase activity adjusted according to β-galactosidase-indicated transfection efficiency.

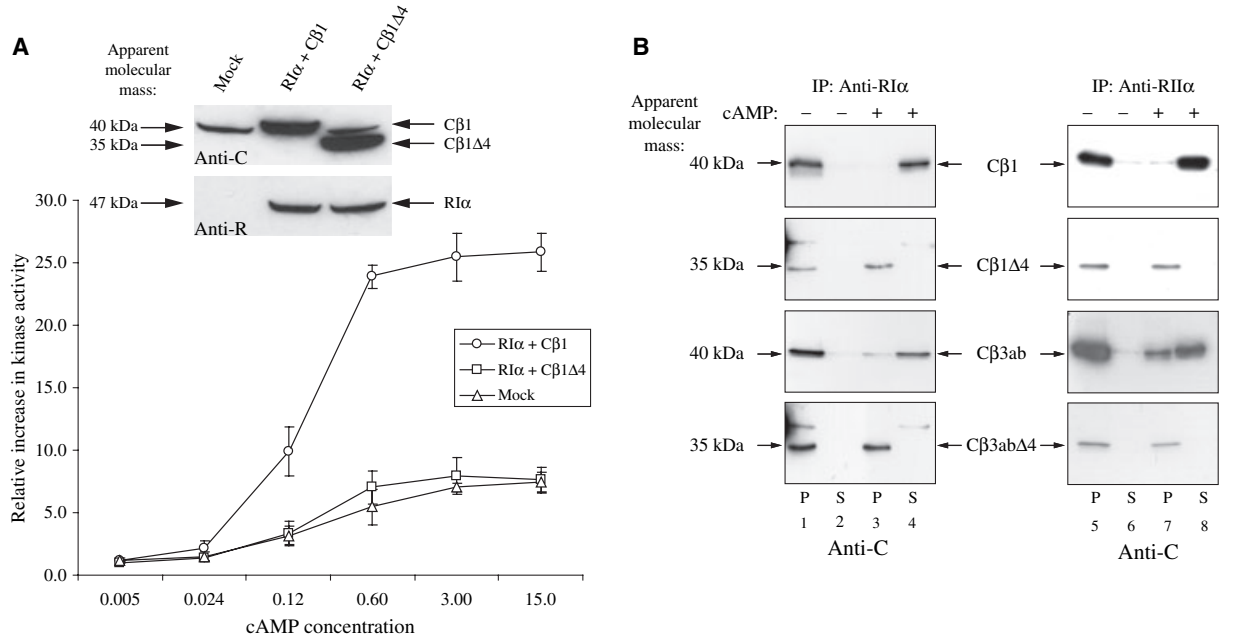


**Fig. 7.** CβΔ4 interaction with the R subunit is cAMP-insensitive. (A) Cell extracts of 293T cells co-transfected with RIα and Cβ1, RIα and Cβ1Δ4 or mock transfected were analyzed by immunoblotting using an RIα antibody [34] and a pan-C antibody (inserts). Immunoreactive PKA C subunits of approximately 40 kDa are recognized in all samples whereas a C subunit 35 kDa band is recognized in the Cβ1Δ4 transfected cells. In addition, transfected RIα subunits of approximately 47 kDa are also recognized. Apparent molecular masses are indicated by arrows. The cell extracts were monitored for PKA-specific kinase activity against kemptide using γ-[$^{32}$P]ATP and increasing concentrations of cAMP. Relative increase in kinase activities were compared to PKA activity in mock transfected cells and are presented as the mean ± SEM from three representative experiments. (B) 293T cells co-transfected with RIα or RIIα and one of the C subunits Cβ1, Cβ1Δ4, Cβ3ab and Cβ3abΔ4 were homogenized and cell lysates immunoprecipitated with anti-RIα (left panel) or anti-RIIα (right panel) sera depending on the transfected R subunit, or irrelevant IgG (not shown). Immunoprecipitated proteins were untreated (–) or treated (+) with 1 mM cAMP, and the pellets (P) and the supernatants (S) were analyzed by immunoblotting using a pan-C antibody. Note that none of the CβΔ4 variants are released neither from RIα nor RIIα by 1 mM cAMP. Arrows on the left indicate the apparent molecular weight and arrows in the middle indicate C subunit identity.

supernatants analyzed for C subunit immunoreactive proteins. This demonstrated that Cβ1 and Cβ3ab are released into the supernatant fraction after cAMP treatment (Fig. 7B, lanes 4 and 8) implying that they are released from the R subunit. This was not the case with Cβ1Δ4 and Cβ3abΔ4 which remained in the pellet fraction after treatment with saturating concentrations of cAMP (Fig. 7B, lanes 3 and 6), implying that their association with the R subunit is insensitive to cAMP. Control experiments were performed by immunoprecipitating with irrelevant IgG (not shown). Taken together, these findings demonstrate that CβΔ4 subunits form cAMP insensitive PKA type I and type II holoenzymes.

## Discussion

The human genome is now completely sequenced and the number of protein-coding genes is estimated to between 20 000 and 25 000 [22]. Humans generate a considerably larger number of proteins than the number of available genes; post-translational modifications, RNA editing, alternative polyadenylation and multiple start sites of transcription contribute to generating diversity, but alternative splicing is the major mechanism by which this is achieved [23]. In the present study, we have identified and characterized six novel PKA Cβ subunits that lack the sequence encoded by the exon 4 of the PKA Cβ gene. The novel Cβ variants were designated CβΔ4. They were identified in NT2-N cells, human and Rhesus monkey brain, but not in human PBL or mouse brain, suggesting that skipping of exon 4 in the Cβ gene may only take place in nerve cells of higher primates. The CβΔ4 variants were devoid of catalytic activity both *in vitro* and *in vivo*. Moreover, CβΔ4 variants associated with RI and RII in a cAMP-insensitive fashion.

Alternative splicing is an excellent means for diversifying the properties of a protein and can give each splice variant specific and fine-tuned characteristics.

The Cβ gene has been shown to encode a variety of splice variants that are differentially spliced at the N-terminal end [5,6]. Our experiments demonstrated the presence of six Cβ mRNAs produced by the deletion of the 99 bases encoded by exon 4. This type of alternative splicing may be restricted to the Cβ gene because we were unable to detect exon skipping for Cα and it has not been described for any of the other PKA genes.

In an attempt to investigate the distribution of the novel Cβ splice variants, we developed a screening method that enabled us to specifically detect low levels of CβΔ4 mRNAs. The method takes advantage of a unique *Ssp*I restriction site in the Cβ exon 4 sequence. By using this method, we found that the CβΔ4 variants may be restricted to nerve cells because they were not identified in human PBL despite the fact that these cells express relatively high levels of the Cβ variants Cβ1 and Cβ2 [5,17,18]. Nevertheless, based on these results, we cannot rule out the possibility that CβΔ4 variants may be expressed at low levels in other Cβ expressing tissues and an expressed sequence tag clone representing CβΔ4 in placenta (accession number DA854574) indicates that this phenomenon may not be restricted to nerve cell tissues. However, all other human CβΔ4 expressed sequence tags originated from brain (accession numbers DA495136, DA217168, DA216689, DA126431, DA502730, DC305863 and DC310086) and several of the CβΔ4 variants contained sequences encoded by the exons a, b and c in the Cβ gene that are only transcribed in nerve cells [6]. In addition, the brain is the tissue with the highest frequency of alternative splicing by exon skipping [24]. This prompted us to search for CβΔ4 variants in the brain of other species. By applying our screening method, we detected CβΔ4 variants in Rhesus monkey but not in mouse brain cDNA. In the latter species, several studies demonstrate at least three Cβ splice variants exist [20,25,26]. Based on these results, it may be hypothesized that Cβ exon 4 skipping is a nerve cell specific phenomenon taking place in the brain of higher primates. However, as stated above, we cannot completely rule out the possibility that extremely low levels of CβΔ4 variants are expressed in mouse brain as well.

When we positioned the exon 4 encoded amino acids into the Cα 3D protein structure [27], we found that the sequence encodes a crucial component of the catalytic cleft. Based on this information, we expected that all C subunits lacking this sequence would have altered catalytic activity. Indeed, all *in vitro* as well as *in vivo* testing of expressed CβΔ4 variants revealed that they were incapable of phosphorylating the two well-characterized PKA substrates, kemptide and histone H1 [28–30], as well as inducing a CRE-regulated promoter regulating a luciferase reporter gene. Together, these results suggest that lack of the exon 4 induces a structural change in the catalytic cleft, rendering the CβΔ4 variants inactive.

When stimulating with increasing concentrations of cAMP or washing with high concentrations of cAMP after immunoprecipitation with anti-RI and anti-RII sera of cells co-transfected with the respective R subunit and either full-length or exon 4-lacking C subunits, it appeared that the association of CβΔ4 variants with the R subunits is insensitive to cAMP. Whether cAMP insensitive CβΔ4 results from an aberrant splicing error without biological significance, or whether expression of exon 4-lacking C subunits contributes to a more complex cAMP and PKA signalling pathway in higher primates compared to other species, remains to be seen. It should, however, be mentioned that neuronal expression of RIβ represents a means of changing PKA holoenzyme sensitivity to cAMP [31]. This is probably not the case for CβΔ4 because it did not alter the cAMP sensitivity of the endogenous holoenzymes in 293T cells even when expressed at higher levels compared to endogenous C, as judged by the levels of immunoreactive protein. We also conclude that the association and dissociation of the endogenous holoenzymes appeared to be unaffected by the co-expression of RIα and Cβ1Δ4. This is suggestive of a continuous and complete association of newly synthesized RIα and Cβ1Δ4, further implying that Cβ1Δ4 does not compete to displace full-length C from the endogenous PKA holoenzymes. Again, this suggests that free CβΔ4 does not have a higher affinity for the R subunits than for the full-length C subunits. Finally, this may indicate that CβΔ4 variants can regulate the availability of newly synthesized R and thus influence PKA signalling *in vivo* by regulating cAMP sensitivity.

## Experimental procedures

### Cell cultures

293T cells were maintained in RPMI 1640 (Sigma-Aldrich, Oslo, Norway) containing 10% fetal bovine serum (Sigma-Aldrich), 2 mM L-glutamine (Sigma-Aldrich), 0.1 mM non-essential amino acids (Gibco BRL, Invitrogen, Oslo, Norway), 1 mM sodium pyruvate (Gibco BRL) and penicillin-streptomycin (Sigma-Aldrich) 50 $U \cdot mL^{-1}$ and 50 $\mu L \cdot mL^{-1}$, respectively. The cells were subcultured by splitting in a ratio of 1 : 5 three times a week.

NT2 cells were maintained in DMEM (Sigma-Aldrich) containing 10% fetal bovine serum (Sigma-Aldrich), 2 mM

L-glutamine (Sigma-Aldrich) and penicillin-streptomycin (Sigma-Aldrich) 50 $U \cdot mL^{-1}$ and 50 $\mu L \cdot mL^{-1}$, respectively. The cells were subcultured by trypsination and differentiated by retinoic acid to neuronal cells as described earlier [6,19].

## RT-PCR

Total RNA from NT2-N cells was isolated using the RNeasy Mini Kit (Qiagen, Qiagen Nordic, Solna, Sweden). One μg of NT2-N total RNA was used to make first-strand cDNA by the Reverse Transcription system (Promega, Madison, WI, USA), which was used as template in PCR reactions with the human Cα and Cβ common primer pairs and the Cβ splice variant specific primer pairs listed in Table 1 and Fig. 1A (all from Sigma-Genosys, The Woodlands, TX, USA). PCRs were run with the following cycle conditions: 95 °C for 2 min; 95 °C for 30 s, 60 °C for 30 s, 72 °C for 2 min (30 cycles if not otherwise specified in the figure) and 72 °C for 10 min. Amplification of full-length Cβ and CβΔ4 was achieved with upper primers listed in Table 1, but with lower primer 5′-CCTTCCCTTCAAA TATCACGTAGC-3′ and under the conditions: 94 °C for 1 min; 94 °C for 30 s, 55° for 30 s, 72 °C for 3 min (30 cycles) and 72 °C for 5 min. All PCR products were subjected to 1% agarose gel electrophoresis with ethidium bromide (0.25 $\mu g \cdot \mu L^{-1}$) in TBE buffer. The NT2-N cell PCR products were cloned into the TOPO TA vector pCR2.1 (Invitrogen) and sequenced (Medigenomix GmbH, Martinsried, Germany).

Whereas cDNA from human PBL was prepared by RNA isolation and reverse transcription as described above, cDNA (2.5 $ng \cdot \mu L^{-1}$) from human fetal brain, human adult hippocampus, cerebral cortex and amygdala was purchased from BioChain Institute (Hayward, CA, USA) as PCR Ready First strand cDNA. Total RNA from human adult brain (1.1 $\mu g \cdot \mu L^{-1}$) was purchased from Stratagene (La Jolla, CA, USA) and used with the Reverse transcription system (Promega). In all cases, cDNA was PCR amplified using the Cβ common primers and the results were analysed by agarose gel electrophoresis.

## Screening for Cβ variants lacking exon 4

NT2-N cell, human PBL, human brain and mouse brain cDNA was obtained as described above and Rhesus monkey cDNA was purchased from BioChain Institute. The cDNAs were used as templates in PCRs using the Cβ common primers for the respective species (Table 1). PCR conditions were: Cβ common human: 95 °C for 2 min; 95 °C for 30 s, 60 °C for 30 s, 72 °C for 2 min (20 cycles) and 72 °C for 10 min; Cβ common Rhesus monkey: 94 °C for 2 min; 94 °C for 30 s, 60 °C for 30 s, 72 °C for 2 min (20 cycles) and 72 °C for 10 min; Cβ common mouse: 95 °C for 1 min; 95 °C for 30 s, 60 °C for 30 s and 72 °C for 1 min (20 cycles) and 72 °C for 5 min. Five μL of the PCR mixtures were incubated with *Ssp*I (human and monkey cDNA) or *Pst*I (mouse cDNA) at 37 °C overnight and re-amplified under identical conditions, except that the number of cycles was increased to 35. The resulting fragments were analyzed by agarose gel electrophoresis. If restriction digestion was insufficient, as judged by the intensity of the different bands, the mixture was re-digested and re-amplified under identical conditions.

## Generation of expression vectors

C subunit expression plasmids: NT2-N cDNA was used as template to clone the different Cβ splice variants (Pfu Ultra system; Stratagene). Upper primer 5′-CACCGCCG CCACCATGGGATTGTCACGCAAATCATCAGATGC ATCT-3′ and lower primer 5′-TTAAAATTCACCA AATTCTTTTGCACATT-3′ yielded Cβ3ab and Cβ3abΔ4, distinguished by different migration in a 1% agarose gel. The PCR products were cloned into pENTR D-TOPO (Invitrogen). Cβ1 was cloned by the same method, but by using upper primer 5′-CACCGCCGCCACCATGGGG AACGCGGCGACCG-3′. The inserts were transferred to the mammalian expression vector pEF DEST51 (Invitrogen). Cβ1Δ4 was created by deletion of exon 4 from Cβ1 in pENTR D-TOPO (ExSite mutagenesis kit; Stratagene) with upper primer 5′-GATAATTCTAATTTATACATGGT-3′ and lower primer 5′-CTTCTGCTTATCTAAGATCTTCA-3′ and further recombined into pEF DEST51 (Invitrogen).

R subunit expression plasmids: A pENTR 221 vector with RIα insert (clone ID: IOH25740 PRKAR1A; Invitrogen) was recombined into pEF DEST51 (Invitrogen). RIIα in vector pBluescriptSK + [32] was transferred to pExchange 6A (Stratagene) by *Eag*I and *Not*I restriction enzyme cutting followed by ligation.

## Phosphotransferase assay

293T cells were either mock transfected (Lipofectamine 2000 only; Invitrogen), transfected with Cβ1, Cβ1Δ4, Cβ3ab or Cβ3abΔ4 alone, or co-transfected with Cβ1 and RIα or Cβ1Δ4 and RIα. After 20–24 h, the cells were harvested, washed 3 × NaCl/Pi and lysed for 30 min in 50 mM Tris pH 7.4 containing 0.5% Triton X-100, 100 mM NaCl, 5 mM EDTA, 50 mM NaF, 50 mM NaPP, 1 mM polymethanesulfonyl fluoride, 1 mM $Na_3VO_4$ and protease inhibitor cocktail (Sigma-Aldrich). Lysates were cleared by centrifugation at 16 000 ***g*** for 30 min at 4 °C and protein concentration determined (Bradford protein assay; Bio-Rad Laboratories Ltd, Hemel Hempstead, UK). The samples were adjusted to equal protein concentrations. PKA phosphotransferase activity was measured against the substrates kemptide (Leu-Arg-Arg-Ala-Ser-Leu-Gly, Sigma-Aldrich) and histone H1 (Sigma-Aldrich) using γ-[$^{32}$P]ATP (Amersham Biosciences, Oslo, Norway) in a

reaction mixture described by Roskoski *et al.* [33]. Ten μL of cell extract was incubated in the reaction mixture at 30 °C for 9 min and the reaction stopped by spotting onto P81 phosphocellulose paper (Whatman, Clifton, NJ, USA) and washed in 75 mM phosphorus acid 4 × 15 min at room temperature. The filters were washed once for 10 min in 96% ethanol and air dried. Phosphotransferase activity was measured by liquid scintillation in 3 mL of Opti-fluor (Packard BioScience, PerkinElmer, Waltham, MA, USA).

### Luciferase reporter assay

293T cells were transfected with a CRE-luciferase reporter plasmid, a β-galactosidase control plasmid and the appropriate C subunit expression vector using Lipofectamine 2000 (Invitrogen). Cells were harvested and lysed in Reporter lysis buffer (Promega) by vortexing. Cell debris was pelleted by centrifugation at 16 000 ***g*** for 3 min. Ten μL of lysate was mixed with 100 μL of luciferase assay mix [470 μM luciferin (SynChem Inc., Des Plaines, IL, USA), 0.1 mM EDTA, 3.74 mM $MgSO_4$, 20 mM tricine, 33.3 mM dithiothreitol, 530 μM ATP (Boehringer Ingelheim GmbH, Ingelheim, Germany), 270 μM coenzyme A (Boehringer), pH 7.8] and the emission of photons was measured in a luminometer (Turner Designs, Sunnyvale, CA, USA). The β-galactosidase level in each sample was estimated by comparison to a β-galactosidase standard curve to adjust luciferase activity in relation to the transfection efficiency.

### Immunoprecipitation

293T cells were co-transfected with C and R subunit expression plasmids (Lipofectamine 2000, Invitrogen) were harvested after 20–24 h, washed 3 × NaCl/Pi and resuspended in immunoprecipitation buffer (150 mM NaCl, 50 mM Tris pH 7.4, 0.5% Triton X-100, 1 mM polymethanesulfonyl fluoride, 1 mM $Na_3VO_4$ and protease inhibitor cocktail; Sigma-Aldrich), vortexed thoroughly and incubated on ice for 30 min. Lysates were cleared by centrifugation at 16 000 ***g*** for 30 min at 4 °C, protein concentrations determined (Bradford protein assay; Bio-Rad) and samples adjusted to equal protein concentrations. Lysates were pre-cleared with Protein G coated beads (Dynabeads; Invitrogen). Irrelevant rabbit IgG, anti-RIα [34] or anti-RIIα rabbit IgG [35] sera (1 : 100 dilutions) were added to the proper samples and incubated with rotation at 4 °C overnight followed by incubation with Protein G beads for 1 h at 4 °C. Beads were pelleted and washed three times with immunoprecipitation buffer. The pellets were resuspended in immunoprecipitation buffer with or without 1 mM cAMP (Sigma-Aldrich) for 5 min followed by SDS/PAGE for immunoblot analysis of the pellets and supernatants.

### Immunoblotting

Cell lysates separated by SDS/PAGE (Bio-Rad) were transferred to polyvinylidene difluoride membranes (Millipore, Oslo, Norway) followed by blocking in 5% skimmed milk powder in NaCl/Tris with 0.1% Tween-20 (TBST) for 1 h at room temperature or overnight at 4 °C. The blot was then incubated at room temperature with primary antibody $PKA_C$ (BD Transduction Laboratories, cat # 610981; BD Norge AS, Trondheim, Norway) or anti-RIα serum [34] diluted 1 : 500 in TBST for 1 h, washed 6 × 10 min in TBST and further incubated with horseradish peroxidase-conjugated secondary antibodies (MP Biomedicals, Irvine, CA, USA) diluted 1 : 2000 in TBST. After a final wash of 6 × 10 min, immunoreactive proteins were visualized using SuperSignal® West Pico Chemiluminescent (Pierce Biotechnology, Rockford, IL, USA).

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## Supplementary material

The following supplementary material is available online:

**Fig. S1.** Comparison of human and Rhesus monkey PKA Cβ amino acid sequence.

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