Identification of Critical Determinants for Autoinhibition in the Pseudosubstrate Region of Type Iα cAMP-dependent Protein Kinase*

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The consensus substrate site for cAMP-dependent protein kinase (PKA) is Arg-Arg-Xaa-Ser(P)-Xaa and the autoinhibitory domain of the PKA type Iα regulatory subunit (RI subunit) contains a similar sequence, Arg92–95. Arg-Arg-Arg-Gly-Ala-Ile-Ser-Ala-Glu. The italicized amino acids form a putative pseudosubstrate site (Ser is replaced with Ala), which together with adjacent residues could competitively inhibit substrate phosphorylation by the PKA catalytic subunit (C subunit). The present studies determine the contributions of Arg92–95, Ile98, and Glu101 to inhibitory potency. Amino-terminal truncation of RI subunit through Arg95 (Δ1–92) or Arg93 (Δ1–93) had no detectable effect on inhibition of C subunit. Truncation through Arg94 (Δ1–94), or point mutation of Arg95 within truncated mutants (Δ1–93.R95A or Δ1–92.R95A), caused a dramatic reduction in inhibitory potency. Truncation through Arg96 (Δ1–95) had a greater effect than did replacement or deletion of Arg92 or Arg93 alone. Using full-length RI subunit, the inhibitory potency was reduced by replacing Ile98 with Ala, Gly, or Gln, but not by replacing it with Val. The inhibitory potency of RI subunit was unchanged when Glu101 was replaced with Ala or Gln. It is concluded that Arg94, Arg95, and, to a lesser extent, Ile98 are vital constituents of PKA autoinhibition by type Iα R subunit.

cAMP-dependent protein kinase (PKA) is a tetramer comprising a dimer of regulatory subunits (R subunit) and two catalytic subunits (C subunit). In the absence of cAMP, PKA is maintained as an inactive holoenzyme complex (R2C2). cAMP binding to the R subunit causes dissociation of the R and C subunits and subsequent activation of the kinase. There are two major classes of PKA holoenzyme, types I and II, denoted by the R subunit isoform associated with C subunit. Despite numerous differences in the R subunit isoforms, they share the same basic domain structure: a short amino-terminal dimerization domain, an autoinhibitory domain located in the amino-terminal segment of the protein, and two cAMP-binding domains toward the carboxyl-terminal end.

The autoinhibitory domain of the R subunit contains a sequence that mimics the consensus phosphorylation sequence of PKA substrates (Arg-Arg-Xaa-Ser(P)-Xaa). In the type II R subunit (RII subunit), this sequence (Arg-Arg-Val-Ser(P)-Val) is a substrate for C subunit, but in the RI subunit, the phosphorylatable Ser is substituted with a non-phosphorylatable Ala residue to form a pseudosubstrate site (Arg-Arg-Xaa-Ala-Xaa). The pseudosubstrate sequence is believed to interact with the C subunit catalytic site to competitively inhibit substrate phosphorylation.

The phosphorylation or pseudophosphorylation site is designated as the P residue, while residues located amino-terminal or carboxyl-terminal to P are designated as minus or plus residues, respectively (4). The P−3 and P−2 Arg residues are important determinants for phosphorylation of PKA substrates (5, 6). Replacement of either residue in peptide substrate analogs profoundly impairs the phosphorylation of the peptide. The P−3 and P−2 Arg residues are invariant in all known R subunit pseudosubstrate sequences and are required for potent inhibition of C subunit (3, 7, 8). They are also found in the pseudosubstrate sequence of the high affinity, heat-stable protein kinase inhibitor of PKA, PKI (Arg-Arg-Gln-Ala-Ile) (9). The importance of these two Arg residues in PKI has also been well documented using peptide analogs (10, 11), and more recently by co-crystallization of C subunit with the peptide derived from the inhibitory segment of PKI, PKI(5–24) (4, 12).

Although many natural substrates for PKA conform to the consensus sequence of Arg-Arg-Xaa-Ser(P)-Xaa, many others contain only a single basic residue at either P−3 or P−2, and/or have additional basic residues more amino-terminal to P (13). Basic residues are frequently found at the P−4, P−6, or both positions in PKA substrates (13), yet other substrates contain a cluster of four basic residues at the P−2 through P−5 positions (14–16). Both P−4 and P−6 basic residues are important recognition factors for phosphorylation of the peptide derived from rabbit skeletal muscle phosphorylase kinase β subunit (17). A similar pattern of basic amino acid residues is noted in the PKA regulatory proteins. A P−6 Arg residue is present in the RII subunit substrate site and in the PKI pseudosubstrate site, while the RI subunit has a cluster of four Arg residues at the P−2 through P−5 positions. The PKI P−6 Arg is crucial for potent inhibition of C subunit (4, 11). The contribution made toward C subunit inhibition by the RII subunit P−6 Arg residue, or the RI subunit P−4 and P−5 Arg residues, has not been investigated previously. Based on the evidence above, it is possible that each of the four Arg residues...
The oligonucleotides are complementary to the cDNA coding strand except for the bold and underlined nucleotides.

| Table I | Synthetic oligonucleotides for RI subunit mutagenesis |
|---------|-------------------------------------------------------|
| 1) Δ1–91 | 5′-GGTCGGCGCGCGCATATGACACGGGATGAG-3′ |
| 1) Δ2–92 | 5′-CCGGCCGCGCGCATATGACACGGGATGAG-3′ |
| 3) Δ1–83 | 5′-GCCCGGCGCGCGCATATGACACGGGATGAG-3′ |
| 3) Δ1–94 | 5′-TGATGGGCACCCGCGCATATGACCGCTCTTA-3′ |
| 4) Δ1–95 | 5′-CGCTAGGGGCGCATATGACACGGGATGAG-3′ |
| 5) Δ1–93.R94A | 5′-GATGGGGGCGCATATGACACGGGATGAG-3′ |
| 6) Δ1–93.R95A | 5′-CGCTAGGGGCGCATATGACACGGGATGAG-3′ |
| 7) Δ1–92.R95A | 5′-CGCTAGGGGCGCATATGACACGGGATGAG-3′ |
| 8) Δ1–93.R95A | 5′-CGCTAGGGGCGCATATGACACGGGATGAG-3′ |
| 9) I96V | 5′-GTAGACCTCGGCGATACACGGGATGAG-3′ |
| 10) I98A | 5′-GTAGACCTCGGCGATACACGGGATGAG-3′ |
| 11) I98G | 5′-GTAGACCTCGGCGATACACGGGATGAG-3′ |
| 12) I98Q | 5′-GTAGACCTCGGCGATACACGGGATGAG-3′ |
| 13) E101A | 5′-GGTGAGTACACGGGATGAG-3′ |
| 14) E101Q | 5′-GGTGAGTACACGGGATGAG-3′ |

in the pseudosubstrate region of RI subunit interacts with an acidic residue(s) in the active site of C subunit.

The P-1 residue is frequently, but not exclusively, a large hydrophobic residue in PKA substrates (13). Studies with synthetic peptide substrates in PKA substrates (13). Studies with synthetic peptide substrates in PKA substrates (13). Studies with synthetic peptide substrates in PKA substrates (13). Studies with synthetic peptide substrates in PKA substrates (13). Studies with synthetic peptide substrates in PKA substrates (13). Studies with synthetic peptide substrates in PKA substrates (13). Studies with synthetic peptide substrates in PKA substrates (13). Studies with synthetic peptide substrates in PKA substrates (13). Studies with synthetic peptide substrates in PKA substrates (13). Studies with synthetic peptide substrates in PKA substrates (13). Studies with synthetic peptide substrates in PKA substrates (13). Studies with synthetic peptide substrates in PKA substrates (13). Studies with synthetic peptide substrates in PKA. The RI subunit cDNA was subcloned into pT7-7 as described previously (28) to create pT7RNI and pT7R, pT7RNI, an intermediate vector, contained the entire RI subunit cDNA, which was out-of-frame. pT7R was the wild type (WT) RI subunit bacterial expression vector in which the RI subunit start codon was mutated to a NdeI site to allow direct in-frame fusion with the pT7-7 start codon, also encoded by a NdeI restriction site.

KSI-IR was created for use in oligonucleotide-directed mutagenesis by subcloning the 1118-base pair (bp) Sad/SalI fragment of pT7RNI (encoding all but 52 base pairs from the 5′ end of the RI subunit cDNA) into the Sad/SalI fragment (2882 bp) of the M13-derived phagemid, pBluescript KSI-IR. Synthetic oligonucleotides 1–13 (Table 1) were used to create KSI-IR by site-directed mutagenesis using the Kunkel method (29). Oligonucleotides 5–8 were used to create the KSI-IR sequence in the 5′ side of the Arg101 codon, Arg230, Arg234, Arg236, and Gly236 codons to a NdeI site (CATATG). Oligonucleotides 6–8 were used to create a NdeI site 5′ to the Arg235 or Arg240 codons, and to mutate the codons for Arg235 (CGA) or Arg240 (GGC) to Ala (GCT) in KSI-IR. Introduction of a NdeI restriction site allowed for direct in-frame fusion with the NdeI restriction site encompassing the pT7R start codon, thus deleting the 5′ segment of the RI subunit cDNA. Oligonucleotides 9–13 were used to mutate the Ile65 codon to Val (GTT), Ala (GCT), Gly (GTT), and Glu (GAG), and the Glu101 codon to Ala (GCT) in KSI-IR. A 700-bp PstI fragment, released from the previously described pUC13-based RI subunit expression vector (pUC13R) (30), was subcloned into the PstI site of M13mp8 to create M13.PstR. Oligonucleotide 14 was used to mutate the Glu101 codon (GAG) to Glu (GAA) in M13.PstR using a commercially available mutagenesis kit (Oligonucleotide-directed in Vitro Mutagenesis, version 2, code RVN, Amersham).

Functional pT7R expression vectors encoding truncated RI subunit cDNA (Δ1–91, Δ1–92, Δ1–93, Δ1–94, Δ1–95, Δ1–93.R94A, Δ1–92.R95A, and Δ1–93.R95A) were created by subcloning the 585–596-bp NdeI-EcoRI fragments from KSI-IR mutants 1–8, into the large NdeI-EcoRI fragment of pT7R. The 769-bp PvuII-EcoRI fragments were isolated from KSI-IR mutants 9–13 and subcloned into the large PvuII-EcoRI fragment of pT7R to create functional mutant pT7R expression vectors encoding full-length RI subunits. Bacteria expression was performed as described in Ref. 28, except
that the media contained 0.1 mg/ml ampicillin and the cells were pelleted at 5000 × g for 15 min each time. The bacterial pellets were stored at −20 °C until homogenization and were viable for more than 1 year when stored in this manner. Initially, the expression cultures yielded ~3–5 mg of RI subunit/liter of culture (5-h induction). Due to the low expression levels, aliquots were frozen in aliquots of 35.5 ml HCl (2–3) to release cyclic nucleotide. Later increased to 36 h, yielding 10–80 mg of RI subunit/liter culture.

Purification of Recombinant RI Subunits—Bacteria were disrupted as described previously (31) except that the homogenization buffer was 10 mM potassium phosphate, pH 6.8, 1 mM EDTA, 2 mM β-mercaptoethanol (KPEM) plus 50 mM benzamidine (KPEM2B) and 2.3 mM 3′,5′-cytidine monophosphate (cIMP). The RI subunit in the equilibrium exchange reaction. A 500-fold molar excess of unlabeled cAMP was used in the dissociation reaction.

Inhibition of C Subunit by Native, WT, and Mutant RI Subunits—Immediately before use, C subunit was diluted in Dilution Buffer (50 mM potassium phosphate, pH 6.8, 0.1 mM dithiothreitol, and 1 mg/ml bovine serum albumin (BSA)). A final concentration of 21 pm C subunit was precipitated with varying concentrations of WT or mutant RI subunit (in KPEM) in Kinase Mix (20 mM Tris, pH 7.4, 20 mM magnesium acetate, 0.1 mM ATP, 0.5 mg/ml BSA, and 0.1 mM isobutylmethylxanthine) for 15–30 min at 30 °C. The protein kinase assay was initiated by the addition of a final concentration of 81 μM Kemptide, 0.071 mM ATP, and [γ-32P]ATP (1000 cpm/pmol). The total assay volume was 35 μl. Following a 60–120-min incubation at 30 °C, the reaction was terminated by spotting 20 μl of reaction mixture onto P-81 phosphocellulose paper and immediately placing the papers into 75 mM phosphoric acid. The P-81 paper was washed in minimum of five times in 75 mM phosphoric acid, dried, and counted in 10 ml of non-aqueous scintillant, or counted by Cerenkov radiation.

Preparation of PKA Holoenzyme from WT RI Subunit and Native C Subunit—cIMP-saturated WT RI subunit (3.8 μM) and native C subunit (14.7 μM) were incubated for 30 min at 30 °C in Kinase Mix containing 0.23 mM ATP and 10 mg/ml BSA (final volume = 15 μl). μl of reaction was spotted onto a P-81 phosphocellulose paper and counted for kinase activity (cAMP). The remainder of the reaction was cooled on ice and then loaded onto a Sephadex CM50 column (0.9 × 0.5 cm) equilibrated in KPEM at 4 °C. Approximately 1 ml of KPEM was added once the sample had entered the resin, and 1-drop fractions (~23 μl) were collected. The fractions were assayed for phosphotransferase activity using the peptide substrate Kemptide. cIMP-saturated RI subunit was pooled and concentrated using a Centricon-30 device.

Determination of Stoichiometry for cIMP Bound to RI Subunits—287 μg of WT or mutant RI subunit was denatured at 95 °C for 2 h in the presence of 13.5 μM HCl (2–3) to release cyclic nucleotide. The solution was briefly centrifuged at 15-min intervals during incubation. Following neutralization, the sample was chromatographed at 23 °C on a Sephadex G-25 superfine column (0.9 × 13 cm) equilibrated in KPEM to purify cyclic nucleotide (cAMP). cIMP elution was quantitated using the cIMP extinction coefficient (εmax = 12.3 absorption units at 248 nM) and corrected for cAMP concentration verified to be cIMP by a UV absorbance scan at 220–320 nm.

Protein Sequence Analyses—The NH2-terminal sequence of various proteins was determined by sequential Edman degradation. The analyses were performed by the core sequencing facility at the University of Washington, Seattle, WA and by the Peptide Sequencing and Amino Acid Analysis Shared Resource at Vanderbilt University.

Cyclic Nucleotide Binding and Dissociation Assays—The cyclic nucleotide binding assay was used to identify RI subunit fractions eluted from gel filtration columns and to quantitate purified RI subunits. [3H]cAMP binding activity was measured as described previously (34) except that 1.1 μM [3H]cAMP (2800–5500 cpm/pmol cAMP) was used, and the reaction mixture was diluted with 2.5 ml of ice-cold 10 mM potassium phosphate, pH 6.8, 1 mM EDTA (KPEM) and the tubes were each rinsed with 2.5 ml of KPE. RI subunit was quantitated based on a stoichiometry of 2 mol of cAMP/mol of RI subunit monomer.

[3H]cAMP dissociation assays were performed as described previously (35) using 0.2 μM [3H]cAMP (3 × 103 cpm/pmol) and 4 mM RI subunit in the equilibrium exchange reaction. A 500-fold molar excess of unlabeled cAMP was used in the dissociation reaction.

Preparation of cAMP-saturated Native RI Subunit—Native type Iα RI subunit (purified from bovine lung homogenate that was primarily used for the purification of cGMP-binding, cGMP-specific phosphodiesterase (cG-BPDE) (32) and type Iα PKG (33) or it was purified from rabbit skeletal muscle according to Ref. 34. When using bovine lung homogenate, protein extract was adsorbed to DEAE-cellulose followed by batch elution with 0.1 M NaCl in 20 mM potassium phosphate, pH 6.8, 1 mM EDTA, and 0.5 mg/ml BSA. After washing with 2.5 ml of buffer, the protein solution was diluted into 19 ml of KPEM. The cAMP-specific phosphodiesterase was eluted with 1 M NaCl. The fractions were pooled, concentrated on a 0.9 × 3.5-cm Sephadex G-100 superfine column equilibrated in KPEM containing 0.2 M NaCl. Fractions were assayed for ⍺binding to N5′-H2N-(CH2)5-cAMP Sepharose affinity resin during purification.

Free cyclic nucleotide was removed from the extract by fractionation on a Sephadex G-25 superfine column (2.75 × 26 cm) equilibrated in KPEM. Protein-containing fractions (~70 ml) were collected and clarified by centrifugation at 12,000 × g for 30 min. The extract was chromatographed on a 1.5 × 3.5-cm N5′-H2N-(CH2)5-cAMP Sepharose Equilibrated (in KPEM) at a flow rate of ~1 drop/12 s, or was divided and chromatographed over two columns (1.5 × 3.5 cm and 1.5 × 11.5 cm). In most instances, the flow-through was passed over the column(s) two or three times. RI subunits were isolated by the method of 0.05 M NaCl at 4°C. Types I and II RI subunit were each rinsed with 2.5 ml of KPE. RI subunit was quantitated based on a stoichiometry of 2 mol of cAMP/mol of RI subunit monomer.

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Mutagenesis of cAMP Kinase Autoinhibitory Domain

TABLE II
Truncations and mutations in the pseudosubstrate region of the type Iα R subunit

| Wild type | Δ1–91 | Δ1–92 | Δ1–93 | Δ1–94+ | Δ1–93.R95A | Δ1–92.R95A | Δ1–95 |
|-----------|-------|-------|-------|--------|------------|------------|-------|
|           | 92    | 93    | 94    | 95     | 96         | 97         | 98    | 99    | 100 | 101 |
| I98V      |       | Arg   | Arg   | Arg    | Gly        | Ala        | Ile   | Ser   | Ala | Glu |
| I98A      |       | Arg   | Arg   | Arg    | Gly        | Ala        | Ile   | Ser   | Ala | GLY |
| I98Q      |       | Arg   | Arg   | Arg    | Gly        | Ala        | Ile   | Ser   | Ala | GLN |
| E101A     |       | Arg   | Arg   | Arg    | Gly        | Ala        | Ile   | Ser   | Ala | ALA |
| E101Q     |       | Arg   | Arg   | Arg    | Gly        | Ala        | Ile   | Ser   | Ala | GLN |

The residue number is indicated above the sequences. The NH₂-terminal residue is denoted by a superscript 1 in the truncated RI subunits, and the mutated residues are capitalized and underlined.

RESULTS AND DISCUSSION

Amino-terminal truncation and site-specific mutants (Table II) of the RI subunit were employed to investigate the contributions that conserved residues within and near the pseudosubstrate region make toward the potency with which RI subunit inhibits C subunit kinase activity. The individual weights of 35,000 on SDS-PAGE, but were calculated to have molecular weights of ~31,790–32,414 by amino acid sequence. The purified WT, E101Q, and all truncated RI subunits were subjected to amino acid sequence analysis to verify the predicted primary structure at the amino termini. The initiator Met was present in proteins in which the penultimate residue was Arg, but it was absent from those in which this residue was Ala (WT, E101Q, Δ1–94+), and Gly (Δ1–95). These results were consistent with the previous finding that cleavage of the initiator Met from proteins during post-translational modification is dependent on the size of the adjoining residue (38). Protein expressed from proteins during post-translational modification is dependent on the size of the adjoining residue (38).

Confirmation of the Structural Integrity of Recombinant RI Subunits

All segments of RI subunit cDNA subjected to a mutagenesis reaction were sequenced to verify the integrity of the mutation and subcloning sites before overexpression in bacteria. The recombinant RI subunits were purified to apparent homogeneity as shown in Fig. 1 (see “Experimental Procedures”). Consistent with previous results (36), the full-length RI subunit migrated at Mₑ ~ 49,000 when analyzed by SDS-PAGE, considerably larger than the molecular weight calculated by the amino acid sequence for bovine skeletal muscle RI subunit (42,804) (37). The truncated RI subunits migrated at Mₑ ~35,000 on SDS-PAGE, but were calculated to have molecular weights of ~31,790–32,414 by amino acid sequence. The purified WT, E101Q, and all truncated RI subunits were compared to that of WT. Fig. 2 displays the characteristic biphasic cAMP dissociation (exchange) pattern of WT, and those of representative mutants: Δ1–94, E101A, and I98A.
In theory, a quantitative measure of C subunit inhibition would be possible if the RI subunit preparation were bound with a low affinity cyclic nucleotide instead of with cAMP. Thus, cAMP was replaced with cIMP, which exhibits an affinity that is ~20-fold lower than that of cAMP for the type Ia RI subunit cyclic nucleotide binding sites (52). Since expression of RI subunit in E. coli produces the cAMP-saturated form of the enzyme, the exchange of cIMP for cAMP was performed during the preparation of bacterial extracts (see “Experimental Procedures”). This facilitated subsequent purification of RI subunits using N6-H2N-(CH2)2-cAMP Sepharose affinity resin. RI subunit was eluted from the affinity resin with cIMP, and the excess cIMP was separated from RI subunit by molecular sieve chromatography, producing cIMP-saturated recombinant RI subunits (~2 mol of cIMP/mol of RI subunit monomer) (see “Experimental Procedures”). The cIMP-saturated form of type Ia native RI subunit (from bovine lung) was also prepared. In this case, the purified preparation of cIMP-saturated native RI subunit was incubated with cIMP to allow exchange, followed by molecular sieve chromatography (see “Experimental Procedures”). The retention of cIMP by the RI subunits was unexpected since removing bound cAMP by exchanging for a lower affinity cyclic nucleotide (e.g. cGMP), followed by a procedure such as ion exchange chromatography (30), molecular sieve chromatography (41) or dialysis (35) to remove the lower affinity cyclic nucleotide, is a strategy commonly employed by investigators, including our own laboratory (30).

Unlike the cAMP-saturated RI subunit, the cIMP-saturated WT and native RI subunits completely inhibited C subunit in a concentration-dependent manner; both WT and native proteins exhibited similar I_{C50} values, 0.36 nM and 0.40 nM, respectively (Fig. 3C). The I_{C50} values were determined by Hill plots and represented the concentration of RI subunit required to inhibit C subunit kinase activity by 50%. The I_{C50} concentrations introduced into these assays via the R_{2cAMP} complex (50 nM) were calculated to be well below the K_{D} of cIMP for WT holoenzyme, which was ~750 nM as determined by a Hill plot (data not shown). The inhibition curves for selected cIMP-saturated RI subunit mutants are illustrated in Fig. 4; complete or nearly complete inhibition of C subunit was achieved with each of the RI subunit mutants.

Inhibition of C Subunit by cIMP-Saturated RI Subunits

In the absence of cAMP, PKA is maintained as an inactive holoenzyme complex R_{2C} with an affinity of ~0.2 nM between R and C subunit (39). cAMP binding to the R_{2} in the R_{2C} complex decreases its affinity for C subunit by 4–5 orders of magnitude (40–42), resulting in dissociation of the R and C subunits and subsequent activation of the kinase (1) (Reaction 1). A ternary complex involving the R and C subunits and cAMP (exemplified as R_{2cAMP}C_{2}) is formed as an intermediate during both activation and inactivation of PKA (40, 43–45). Other types of ternary complexes (e.g. R_{2cAMP}C) are also formed, but are not shown in this simplified Reaction 1 (46, 47).

\[
\text{R}_{2\text{C}_{2}} + 4\text{cAMP} \rightarrow \text{R}_{2}\text{cAMP}_{4}\text{C}_{2} \rightarrow \text{R}_{2}\text{cAMP}_{4} + 2\text{C}
\]

**Reaction 1**

The forward reaction is exemplified in Fig. 3A, which shows that holoenzyme was activated by cAMP in a concentration-dependent manner. Activation was evident when the cAMP concentration exceeded 2 nM, and holoenzyme was fully activated at ~75–100 nM cAMP. Half-maximal activation of WT holoenzyme \(K_{D}\) was 21 nM as determined by a Hill plot.

The reverse of Reaction 1, inhibition of C subunit by formation of the R_{2C} complex, is facilitated in vitro by the action of cyclic nucleotide phosphodiesterases which degrade cAMP (48, 49). Because of the high affinity with which cAMP is bound to R subunit in vitro, and because of its dissociation-reassociation characteristics, cAMP remains associated with R subunit throughout extensive molecular sieve chromatography, ion exchange chromatography and dialysis (3). In fact, a significant portion (~20%) of intracellular R subunit contains bound cAMP even in the basal state (50). Because much of the in vitro characterization of native R subunit has utilized the cAMP-bound form of the protein, the reverse reaction was initially examined by incubating cAMP-saturated native RI subunit (R_{2cAMP}) with the purified C subunit (Fig. 3B). C subunit was inhibited by RI subunit in a concentration-dependent manner until the concentration of bound cAMP added to the reaction exceeded 2 nM, at which point the inhibition by RI subunit was reversed, presumably because the equilibrium of Reaction 1 shifted back toward the right. These results are consistent with the results presented in Fig. 3A, which showed that the inactive holoenzyme complex predominated until the added cAMP reached 2 nM, whereupon the equilibrium shifted to the right, toward CAMP saturation of the R subunit and dissociation of the holoenzyme complex. The concentration of cAMP in the reaction at the point at which the equilibrium shifted to the right (2 nM) correlated well with the mean equilibrium binding constant \(K_{D}\) of cAMP for RI subunit (~1.4 nM) (51). When the concentration of cAMP in the reaction exceeded the \(K_{D}\) (Fig. 3, A and B), the equilibrium of cAMP binding to RI subunit shifted toward formation of the R_{2cAMP} complex. These considerations demonstrate the technical difficulties involved in quantitating the potency with which R subunit inhibits C subunit kinase activity when using R subunit maintained in the physiological, cAMP-bound form. Therefore, attempts were made to remove cAMP from RI subunit either by exchanging it for a lower affinity cyclic nucleotide or by urea denaturation as described in the following sections.

* E. Poteet-Smith, J. B. Shabb, S. H. Francis, and J. D. Corbin, unpublished observations.
subunit mutants (RcIMP2) that were used to examine the contribution of the amino-terminal segment of RI subunit, and each residue of the Arg92–95 tetrad. The cIMP-saturated truncated RI subunits are represented as RcIMP2 instead of R2cIMP4 since the dimerization domain has been eliminated by truncating the protein. Truncation of the amino-terminal segment of RI subunit through Gly91 (D1–91) did not impair the inhibitory potency of this mutant compared to that of WT. In the crystal structure of the C subunit zPKI-(5–24) complex, the P2Arg forms a hydrogen bond with Glu203, the P23Arg forms hydrogen bonds with Glu127 and Glu331 and the P22Arg hydrogen bonds with Glu230 and Glu170 (4). Since the RI subunit pseudosubstrate region contains four arginine residues (P25, P24, P23, P22), it is possible that each Arg could interact with one or more of these Glu residues in C subunit, and thus contribute to the high affinity interaction with C subunit. However, truncation through Arg92 (P25) or Arg93 (P24) did not detectably impair the inhibitory potency of either of these mutants (D1–92 and D1–93). The importance of the P23 residue (Arg94) was investigated using two RI subunit mutants (D1–94 and D1–94*) which were truncated through Arg94. The inhibitory potency of D1–94 was 17000-fold lower than that of WT, while the D1–94* mutant, which lacks the initiator Met, was 21000-fold less potent than WT. Thus, the initiator Met was

![Figure 3](http://www.jbc.org/)

**Figure 3.** Effect of cAMP and cIMP on the interaction of RI subunit and C subunit. A, holoenzyme was prepared from cIMP-saturated WT RI subunit and native C subunit as described under “Experimental Procedures.” 1 nM WT holoenzyme was incubated with varying concentrations of cAMP in the presence of 20 mM Tris, pH 7.4, 20 mM magnesium acetate, 0.1 mM ATP, 0.5 mg/ml BSA, 0.1 mM isobutylmethylxanthine, and 81 μM Kemptide substrate for 30 min at 30 °C. The reaction was terminated and the amount of Kemptide phosphorylation was determined. Ka was calculated by a Hill plot. B, C subunit (21 pm) was preincubated with increasing concentrations of cAMP-saturated native type Ia R subunit in the presence of 20 mM Tris, pH 7.4, 20 mM magnesium acetate, 0.1 mM ATP, 0.5 mg/ml BSA, 0.1 mM isobutylmethylxanthine for 19 min at 30 °C. The reactions were initiated by the addition of Kemptide substrate, and the incubation proceeded for an additional 60–120 min at 30 °C. The reactions were terminated, and the amount of Kemptide phosphorylation was determined. IC50 values were calculated from Hill plots.

![Figure 4](http://www.jbc.org/)

**Figure 4.** Inhibition of C subunit activity by cIMP-saturated WT and mutant RI subunits. C subunit (21 pM) was preincubated for 15–30 min at 30 °C with varying concentrations of either WT (●), D1–94* (○), Δ1–93 (□), Δ1–95 (△), I98Q (△), or I98G (■) RI subunits as described in Fig. 3B. The reactions were initiated by the addition of Kemptide substrate, and the incubation proceeded for an additional 60–120 min at 30 °C. The reactions were terminated, and the amount of Kemptide phosphorylation was determined. 100% catalytic activity was determined to be ~6 pmol/min/ng of C in the absence of RI subunit and cAMP. Each curve represents the mean of ≥4 assays for the Arg92–95 mutants, 2 assays for the Ile98 mutants, and 31 assays for WT RI subunit.

subunit mutants (RcIMP2) that were used to examine the contribution of the amino-terminal segment of RI subunit, and each residue of the Arg92–95 tetrad. The cIMP-saturated truncated RI subunits are represented as RcIMP2 instead of R2cIMP4 since the dimerization domain has been eliminated by truncating the protein. Truncation of the amino-terminal segment of RI subunit through Gly91 (D1–91) did not impair the inhibitory potency of this mutant compared to that of WT. In the crystal structure of the C subunit zPKI-(5–24) complex, the PKI P2Arg forms a hydrogen bond with Glu203, the P23Arg forms hydrogen bonds with Glu127 and Glu331 and the P22Arg hydrogen bonds with Glu230 and Glu170 (4). Since the RI subunit pseudosubstrate region contains four arginine residues (P25, P24, P23, P22), it is possible that each Arg could interact with one or more of these Glu residues in C subunit, and thus contribute to the high affinity interaction with C subunit. However, truncation through Arg92 (P25) or Arg93 (P24) did not detectably impair the inhibitory potency of either of these mutants (Δ1–92 and Δ1–93). The importance of the P23 residue (Arg94) was investigated using two RI subunit mutants (Δ1–94 and Δ1–94*) which were truncated through Arg94. The inhibitory potency of Δ1–94 was ~17000-fold lower than that of WT, while the Δ1–94* mutant, which lacks the initiator Met, was ~21000-fold less potent than WT. Thus, the initiator Met was

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not detrimental to the interaction of Δ1–94 with C subunit. The requirement for a P–2 Arg (Arg⁹⁵) was examined using the truncated Δ1–93.R95A mutant. Substitution of Arg⁹⁵ with Ala within Δ1–93.R95A profoundly reduced the inhibitory potency of this truncated mutant (−31000-fold). Removal of all of the amino-terminal Arg residues, Δ1–95, reduced the inhibitory potency of RI subunit for C subunit by −54000-fold. This was an additive decrease in effectiveness compared to that observed when Arg⁸⁴ or Arg⁹⁵ were deleted or mutated individually.

These results demonstrated that both Arg⁸⁴ and Arg⁹⁵ are critical determinants for potent inhibition of C subunit, but that neither Arg⁹² nor Arg⁹⁵ is detectably involved. The results also indicated that the amino-terminal 91 residues are not involved in autoinhibition, despite the established role of the PKI residues which correspond to residues 79–87 in RI subunit (4, 18). The results are in agreement with similar studies in full-length RI (8) and RII subunits (7), and with PKI peptide analog studies (10, 11), in which the P–2 position was reduced the IC₅₀ values for the Δ1–95 mutant. Substitution of Ile⁹⁸ with Gly in PKI peptide analogs reduced the inhibitory potency of the peptide 150-fold (18). In the crystal structure of the C subunit-PKI-(5–24) complex, Ile⁹⁸ (P–1) is situated in a hydrophobic pocket on C subunit comprising Leu¹⁸⁸, Pro²⁰², and Leu²⁰⁵, indicating that a P–1 hydrophobic residue is essential to a high affinity interaction between PKI and C subunit (4).

Since a role in C subunit inhibition has not been previously demonstrated for the R subunit P–1 residue, mutants of the RI subunit P–1 residue (198W, 198A, 198G, and 198Q) were used to investigate the importance of aliphatic side chain length and steric constraints on the ability of RI subunit to potently inhibit C subunit. The IC₅₀ values for the cIMP-saturated, full-length RI subunit mutants (R₂cIMP₄) at the P–1 position (Ile⁹⁸) are presented in Table IV. Conservative replacement of Ile⁹⁸ with Val (198V) in full-length RI subunit did not impair the IC₅₀ value of 198V compared to that of WT. When the aliphatic side chain length at the P–1 position was reduced the IC₅₀ values were increased by −5300-fold and 14000-fold for I98A and I98Q, respectively, compared to that of WT. When the side chain length was increased, as in the 198Q mutant, the IC₅₀ value was −4200-fold greater than that of WT. The loss of inhibitory potency was greatest for 198Q, but substitution of Gly at the P–1 position could cause a conformational change that alters the position of other critical residues in the pseudosubstrate region. The increased length of the Gln side chain and its hydrophilic nature are the most probable factors causing the reduced inhibitory potency for the I98Q mutant.

The results suggested that the RI subunit P–1 hydrophobic residue is quite important for a potent interaction with C subunit, and that the length of the aliphatic side chain is crucial. Strict steric constraints at the P–1 site are also indicated by Kemptide analog studies. Substitution of the P–1 residue in Kemptide with Pro (LRRASPG) resulted in a significant reduction in substrate capacity (56), but introduction of N⁵-methyl Leu in place of Leu at the P–1 position resulted in only a moderate loss of substrate capacity (57).

Effects of Substitutions at Ile⁹⁸—The P–1 position is highly conserved as Ile or Val in the autoinhibitory domains of all species of PKA, PKG and in PKI. Substitution of Ile⁹⁸ (P–1) with Gly in PKI peptide analogs reduced the inhibitory potency of the peptide 150-fold (18). In the crystal structure of the C subunit-PKI-(5–24) complex, Ile⁹⁸ (P–1) is situated in a hydrophobic pocket on C subunit comprising Leu¹⁸⁸, Pro²⁰², and Leu²⁰⁵, indicating that a P–1 hydrophobic residue is essential to a high affinity interaction between PKI and C subunit (4). Since a role in C subunit inhibition has not been previously demonstrated for the R subunit P–1 residue, mutants of the RI subunit P–1 residue (198W, 198A, 198G, and 198Q) were used to investigate the importance of aliphatic side chain length and steric constraints on the ability of RI subunit to potently inhibit C subunit. The IC₅₀ values for the cIMP-saturated, full-length RI subunit mutants (R₂cIMP₄) at the P–1 position (Ile⁹⁸) are presented in Table IV. Conservative replacement of Ile⁹⁸ with Val (198V) in full-length RI subunit did not impair the IC₅₀ value of 198V compared to that of WT. When the aliphatic side chain length at the P–1 position was reduced the IC₅₀ values were increased by −5300-fold and 14000-fold for I98A and I98Q, respectively, compared to that of WT. When the side chain length was increased, as in the 198Q mutant, the IC₅₀ value was −4200-fold greater than that of WT. The loss of inhibitory potency was greatest for 198Q, but substitution of Gly at the P–1 position could cause a conformational change that alters the position of other critical residues in the pseudosubstrate region. The increased length of the Gln side chain and its hydrophilic nature are the most probable factors causing the reduced inhibitory potency for the I98Q mutant.

Although neither Arg⁹² (P–5) nor Arg⁹³ (P–4) is required for potent inhibition of C subunit, it is possible that these highly conserved residues make minor contacts with C subunit that would be more evident in the absence of the P–3 or P–2 inhibitory residues (Arg⁸⁴ or Arg⁹⁵). It is also plausible that some flexibility exists in the interaction between pseudosubstrate residues and C subunit that would permit a shift in the spatial location so that the P–4 and P–3 Arg residues could serve the same role as the P–3 and P–2 Arg residues. The P–4 P–3 arrangement of basic residues is seen in naturally occurring PKA substrates such as phosphorylase kinase (β subunit) (54) and glycogen synthase (site 1b) (55). These substrates have a P–4 Lys and a P–3 Arg residue, but do not possess an Arg residue at the P–2 position. The Δ1–92.R95A mutant, in which an Ala residue was substituted for the P–2 Arg, was used to examine the interaction between the RI subunit P–4 P–3 Arg residues and the active site of C subunit. The Δ1–92.R95A mutant was an extremely poor inhibitor of C subunit, comparable to Δ1–93.R95A (Table III), clearly demonstrating that the RI and C subunit interaction is not flexible enough to permit spatial repositioning of the Arg residues by even a single residue.
of WT RI subunit. Despite the highly conserved nature of the P+4 Glu and its proximity to the pseudosubstrate site, Glu\textsuperscript{101} does not appear to play an important role in C subunit inhibition, although it could serve some other function.

**Influence of cIMP on Inhibition of C Subunit by RI Subunits**

Although complete or nearly complete inhibition of C subunit was achieved using each of the cIMP-saturated RI subunit mutants, concentrations as high as 20–50 \( \mu \text{M} \) were required by most RI subunit mutants to inhibit C subunit completely, compared to 0.01 \( \mu \text{M} \) for WT (Fig. 4). C subunit was inhibited in those reactions containing 50 \( \mu \text{M} \) mutant RI subunit (100 \( \mu \text{M} \) cIMP), despite the fact that the cIMP concentration in the reaction was calculated to exceed the \( K_a \) by >100-fold. The results of studies with cIMP-saturated RI subunit (Fig. 3B) would have predicted less C subunit inhibition as the concentration of the R\textsubscript{2}cIMP\textsubscript{4} or R\textsubscript{cIMP2} complex approached the \( K_a \) of cIMP for holoenzyme (750 \( \mu \text{M} \)). When using cIMP-saturated WT RI subunit, cIMP would not be expected to interfere with holoenzyme formation since the assays are performed using very dilute RI subunit (25 \( \mu \text{M} \)) where the concentration of cIMP (50 \( \mu \text{M} \)) would be far below the \( K_a \) (750 \( \mu \text{M} \)). It is suggested that the large rightward shift exhibited in the inhibition curves by certain RI subunit mutants must reflect the following considerations. 1) The decreased affinity between the pseudosubstrate site of the mutant RI subunit and the catalytic site of C subunit causes a rightward shift in the equilibrium of Reaction 1 (toward R\textsubscript{2}cIMP\textsubscript{4} (or R\textsubscript{cIMP2}) and active C subunit); 2) the necessity of using mutant R\textsubscript{2}cIMP\textsubscript{4} (or R\textsubscript{cIMP2}) at concentrations that vastly exceed the mean equilibrium binding constant of cIMP for RI subunit (-28 \( \text{nM} \)) (51, 52) causes a dramatic shift in the equilibrium of cIMP binding to RI subunit (toward formation of the R\textsubscript{2}cIMP\textsubscript{4} (or R\textsubscript{cIMP2}) complex). The second consideration causes a shift of the equilibrium of Reaction 1 even further to the right than that caused by mutation of RI subunit alone, thereby resulting in the high \( IC_{50} \) values determined for the cIMP-saturated RI subunit mutants. This is supported by the observation that the equilibrium of Reaction 1 was progressively shifted to the right when cIMP-saturated Δ1–94\textsuperscript{a} (9.8 \( \mu \text{M} \) RI subunit:19.6 \( \mu \text{M} \) cIMP) and C subunit (21 \( \mu \text{M} \)) were incubated in the presence of increasing concentrations of exogenous cIMP (up to 20 \( \mu \text{M} \)) (data not shown).

**Inhibition of C subunit by R\textsubscript{2}cAMP\textsubscript{4} or R\textsubscript{cIMP2} in the Presence of Excess Cyclic Nucleotide**

Under physiological conditions the PKA holoenzyme complex (R\textsubscript{2}C\textsubscript{2}) is inactive (see Reaction 1). The PKA ternary complex (R\textsubscript{2}cAMP\textsubscript{4}C\textsubscript{2}) is also largely inactive (\( IC_{50} \sim 15 \mu \text{M} \) for type I enzyme) when the reverse reaction of Reaction 1 is measured using pharmacological concentrations of R\textsubscript{2}cAMP\textsubscript{4} and a vast excess of cAMP, such that the RI subunit remains in the R\textsubscript{2}cAMP\textsubscript{4} form (40). Since the \( IC_{50} \) values obtained for several cIMP-saturated RI subunit mutants approximate the \( IC_{50} \) value obtained for this native ternary complex, it was necessary to determine if these values might reflect the inhibited mutant ternary complexes instead of the R\textsubscript{2}C\textsubscript{2} (full-length RI subunit mutants) or RC (truncated RI subunit mutants) holoenzyme complexes.

The \( IC_{50} \) of the WT ternary complex was measured by incubating C subunit with either WT-R\textsubscript{2}cIMP\textsubscript{4} or WT-R\textsubscript{cIMP2} in the presence of a large excess (500 \( \mu \text{M} \)) of cIMP or cAMP (Fig. 5). The WT-R\textsubscript{2}cIMP\textsubscript{4}C\textsubscript{2} and WT-R\textsubscript{cIMP2}C\textsubscript{2} ternary complexes exhibited \( IC_{50} \) values similar to each other (\( IC_{50} \sim 10–12 \mu \text{M} \)), and to that published for the native ternary complex (40). These values were 4–5 orders of magnitude higher than the \( IC_{50} \) value measured for WT holoenzyme (R\textsubscript{2}C\textsubscript{2}) (0.36 \( \mu \text{M} \); Table III). Inhibition of C subunit by a truncated mutant, cIMP-saturated Δ1–93.R95A RI subunit, was also examined in the presence of excess cIMP to measure inhibition of the Δ1–93.R95A ternary complex (R\textsubscript{cIMP2}C). Only 20% of C subunit activity was inhibited by 50 \( \mu \text{M} \) cIMP-saturated Δ1–93.R95A when assayed in the presence of 500 \( \mu \text{M} \) cIMP (Fig. 5), whereas cIMP-saturated Δ1–93.R95A exhibited an \( IC_{50} \) value of 11.2 \( \mu \text{M} \) when assayed in the absence of excess cIMP (Table III). Similarly, only 16% of C subunit activity was inhibited by 5 \( \mu \text{M} \) cIMP-saturated I98A when assayed in the presence of 500 \( \mu \text{M} \) cIMP (data not shown), whereas cIMP-saturated I98A exhibited an \( IC_{50} \) value of 1.9 \( \mu \text{M} \) when assayed in the absence of excess cIMP (Table IV). Assuming that there is no RC or R\textsubscript{cIMP2} complex present when 500 \( \mu \text{M} \) cIMP is included in the reaction, these results suggest that the \( IC_{50} \) values obtained for the cIMP-saturated mutant RI subunits in the absence of excess cyclic nucleotide are indeed a measure of the inhibited RC or R\textsubscript{cIMP2} holoenzyme complexes, and do not reflect the inhibited ternary complex. The results also indicate that the interactions between C subunit and WT- or mutant-R\textsubscript{2}cIMP\textsubscript{4} or mutant-R\textsubscript{cIMP2} are specific interactions and not due simply to the addition of a high concentration of RI subunit; the interaction of C subunit with either Δ1–93.R95A-R\textsubscript{cIMP2} or I98A-R\textsubscript{2}cIMP\textsubscript{4} was profoundly reduced compared to that interaction with WT-R\textsubscript{2}cIMP\textsubscript{4} when measured in the absence or presence of a large excess of cIMP. This also suggests that the same crucial resi-

\begin{table}
\centering
\begin{tabular}{|l|c|c|}
\hline
Regulatory subunit & cIMP-saturated & Cyclic nucleotide-free \\
\hline
Wild type & 0.36 & 0.72 \\
I98V & 0.16 & 2.9 \\
I98A & 1900 & 5000 \\
I98G & 5000 & \\
I98Q & 1500 & \\
E101Q & 0.18 & \\
E101A & 0.59 & \\
\hline
\end{tabular}
\caption{Contribution of Ile\textsuperscript{98} of the RI subunit to inhibition of C subunit by R\textsubscript{2}cIMP\textsubscript{4}C\textsubscript{2} ternary complex. These values were determined in the presence of 500 \( \mu \text{M} \) cIMP (up to 20 \( \mu \text{M} \)) and C subunit (21 \( \mu \text{M} \)).}
\end{table}
Inhibition of C Subunit by Cyclic Nucleotide-free RI Subunit Mutants

For the sake of comparison, it seemed prudent to examine the effect of RI subunit mutation(s) on inhibitory potency using the cyclic nucleotide-free form of RI subunit, whereby the interaction of R and C subunit is measured directly without the interference of cAMP or cIMP (Reaction 2).

\[
\text{R}_2\text{C}_2 \rightleftharpoons \text{R}_2 + 2\text{C} \\
\text{(inactive)} \quad \text{(active)}
\]

**REACTION 2**

Investigators have previously used R subunit that was made cyclic nucleotide-free by urea denaturation (followed by renaturation) to examine the effect of R subunit mutation(s) on the inhibition of kinase activity (8, 58). Since the denatured R subunit has been shown to have the same affinity for C subunit as that of non-denatured R subunit (59), this method was selected for the preparation of cyclic nucleotide-free RI subunit in the present study. R subunits that have been treated with urea, however, have an increased rate of cyclic nucleotide-dissociation from the binding sites (i.e., decreased affinity for cyclic nucleotide) (60). Since the effect on cyclic nucleotide-dissociation was found to be directly related to the length of time that the R subunits were exposed to urea (60), the exposure of the RI subunits to urea was temporally minimized as described under “Experimental Procedures.”

The IC50 values for the cyclic nucleotide-free RI subunits were determined by Hill plots and are summarized in Tables III and IV. Urea treatment of WT RI subunit had minimal effect on the potency of inhibition of C subunit activity compared to that of untreated WT RI subunit (0.72 nM and 0.36 nM, respectively). The IC50 values obtained for the cyclic nucleotide-free RI subunits are specific, but extremely weak inhibitors of C subunit. It is suggested that a more quantitative assessment of the R-C interaction is obtained using RI subunit that is made cyclic nucleotide-free by urea denaturation. Furthermore, considerably less of this RI subunit is required for inhibition than is required when using cIMP-saturated RI subunit, thus facilitating most experimental protocols. Although evidence suggests that the R and C subunit interaction is not impaired as a result of urea treatment of the RI subunit, this possibility cannot be ruled out since urea denaturation alters the affinity of R subunit for cyclic nucleotide (60).

In summary, the results of this study establish that both the P3–1 Arg, and to a lesser extent, the P3–2 Arg residues are critical for potent inhibition of C subunit by RI subunit. Neither the P3–2 Arg, P3–3 Arg, nor the P4 Glu residue has a detectable effect. The results indicate that the interactions between the pseudosubstrate site Arg residues and the catalytic site acidic residues are not flexible enough to tolerate spatial re-orientation of the two required Arg residues from the P3–2 to the P3–3 positions.

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