Substrate Enhances the Sensitivity of Type I Protein Kinase A to cAMP*

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The functional significance of the presence of two major (types I and II) isoforms of the cAMP-dependent protein kinase (PKA) is still enigmatic. The present study showed that peptide substrate enhanced the activation of PKA type I at low, physiologically relevant concentrations of cAMP through competitive displacement of the regulatory RI subunit. The effect was similar whether the substrate was a short peptide or the physiological 60-kDa protein tyrosine hydroxylase. In contrast, substrate failed to affect the cAMP-sensitivity of PKA type II. Size exclusion chromatography confirmed that substrate acted to physically enhance the dissociation of the RIIα and Cα subunits of PKA type I, but not the RIIα and Cα subunits of PKA type II. Substrate availability can therefore fine-tune the activation of PKA type I by cAMP, but not PKA type II. The cAMP-dissociated RII and C subunits of PKA type II reassociated much faster than the PKA type I subunits in the presence of substrate peptide. This suggests that only PKA type II is able to rapidly reverse its activation after a burst of cAMP when exposed to high substrate concentration. We propose this as a possible reason why PKA type II is preferentially found in complexes with substrates undergoing rapid phosphorylation cycles.

The cAMP-dependent protein kinase (PKA)1 is a model for protein kinases because of its universal distribution and its relative simplicity because the catalytic moiety and the auto-inhibitory moiety are on different subunits. The mammalian tetrameric PKA consists of two catalytic (C) subunit monomers and a regulatory (R) subunit dimer. The binding of two molecules of cAMP to each R subunit favors dissociation of the C and R subunits. The kinase exists in two major isoforms with different R subunits, RI and RII, each with α- and β-subforms. The biological significance of the presence of two major isozenzymes is still uncertain. It was early noticed that RI predominated in many cells with rapid proliferation, rapid growth in cell size, or malignantly transformed cells (1–4). More recently it has been shown by homologous recombination that RIIα/−/− mice die in embryonic life, whereas RIIβ/−/−, RIIα/−/−, and RIIβ/−/− mice have less obvious defects, mainly in differentiation of adipose tissue (RIIβ) and neural functions (RIIβ) (5). An important difference between RI and RII is their relative affinity for anchoring proteins that confine PKA type II, and in some cases PKA type I, to subcellular compartments (see Refs. 6 and 7 for recent reviews). Another striking difference is the ability of PKA type II, but not type I, to become autophosphorylated (8–10). The interface between the R and C subunits of PKA is complex. The RI and RII subunits bind in part to non-overlapping areas of the C subunit as well as areas distant from this site (12) and are, through their (pseudo)substrate moiety, believed to displace substrate from the C subunit and thereby inhibit the kinase activity (10, 12). The substrate could displace the R subunit from the substrate binding cleft of the C subunit and thereby facilitate holoenzyme dissociation. In this way PKA could be activated at lower concentrations of cAMP in the presence of substrate.

In the present study we found that PKA type I, but not type II, was sensitized to cAMP by peptide substrate or by macromolecular substrates like tyrosine hydroxylase. This novel observation suggests that the activity of PKA type I is determined not only by the cAMP level but also by the availability of substrate. Substrate inhibited the rate of reassociation of the cAMP-dissociated subunits of PKA type I, but not type II. This suggests that high substrate availability may prolong the duration of the activation of PKA type I after a burst of cAMP.

We have shown previously that physiologically relevant concentrations of cAMP-saturated R subunit of PKA types I and II can form inactive holoenzyme (13). Using physical methods to determine R and C subunit interaction we could confirm this and show that substrate facilitated the dissociation of PKA type I, but not type II. We propose therefore that substrate can selectively enhance PKA type I activity through three mechanisms: by facilitating the activation by low cAMP concentration, by prolonging the activation after a burst of cAMP, and by the dissociation of the holoenzyme at saturating cAMP concentration.

EXPERIMENTAL PROCEDURES

Materials—[2,8-3H]Adenosine3′5′cyclic phosphate and [γ-32P]ATP were from Amersham Biosciences. The heptapeptide LRRASLG (kemptide) was obtained from Bachem AG, Bubendorf, Switzerland. Protein kinase inhibitor peptide (5–24) (14) was from Peninsula Laboratories Inc., San Carlos, CA. Buffer A is 18 mM Hepes, pH 7.2, with 1 mM NaHPO4, 0.4 mM EGTA, 0.1 mM EDTA, 130 mM KCl, 2 mM Mg(CH3COO)2, 0.5 mM dithioerythritol, bovine serum albumin (0.2 mg/ml), and soybean trypsin inhibitor (0.2 mg/ml). The C subunit of PKA type II from bovine heart and recombinant human RI and RII subunits were isolated as described previously (13). Recombinant and native tyrosine hydroxylase (EC 1.14.16.2; tyrosine monoxygenase; l-tyrosine tetrahydropteridine:oxygen dehydrogenase (3-hydroxylase) was kindly provided by Drs. Haavik, Almås, and Flatmark, University of Bergen, Norway. The kinase substrate glutathione S-transferase-SR-RASVGL14–3-3γ was provided by Dr. R. Kleppe, Department of Bio-

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‡ The abbreviations and trivial terms used are: PKA, cAMP-dependent protein kinase; C subunit, catalytic subunit of PKA; RI and RII subunits, regulatory subunits of PKA types I and II, respectively; P-RII, RII subunit autophosphorylated by the C subunit of PKA; kemptide, heptapeptide LRRASLG.
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...preincubated for 30 min in buffer A, and the reaction was initiated by the addition of 0.3 mM ATP or ADP by scintillation proximity assay as described elsewhere (13). The RI and C subunits were preincubated for 30 min in buffer A, and the reaction was initiated by the addition of 0.3 mM ATP or ADP by scintillation proximity assay as described elsewhere (13). The RI and C subunits were preincubated for 30 min in buffer A, and the reaction was initiated by the addition of 0.3 mM ATP or ADP by scintillation proximity assay as described elsewhere (13). The RI and C subunits were preincubated for 30 min in buffer A, and the reaction was initiated by the addition of 0.3 mM ATP or ADP by scintillation proximity assay as described elsewhere (13).

**Phosphotransferase Assays**—The phosphorylation of kemptide was determined essentially as described in Ref. 13. The RI and C subunits were preincubated for 30 min in buffer A, and the reaction was initiated by the addition of [γ-32P]ATP (to a final concentration of 0.3 mM), various concentrations of substrate peptide, and CAMP. The phosphorylation of tyrosine 4-monooxygenase or glutathione S-transferase-SR-RASVGL-14-3-3γ was determined as for kemptide except that the reaction was started by the addition of [γ-32P]ATP just after the addition of substrate protein and the reaction was terminated by adding two volumes of stop solution (1.5 mM ATP, 0.08% SDS, and 60 mM EDTA). Samples (40 μl) of this mixture were spotted on 3MM cellulose filters and subjected to extensive washing in 5% trichloroacetic acid before drying and scintillation counting.

**C Subunit-induced Dissociation of the [3H]cAMP-R Subunit Complex**—The RI or RII subunits were preincubated with [3H]cAMP (1 μM) by incubation for 40 min at 37 °C. Excess (unbound) [3H]cAMP was removed by gel filtration (Sephadex G50) at 2 °C immediately before the assay. The assay was 25 °C and was initiated by the addition of C subunit to 2 mM of R subunit with bound [3H]cAMP in buffer A with 0.6 mM ADP or 1.2 mM AMP. Unlabeled CAMP (2 μM) was present to prevent rebinding of the dissociated [3H]cAMP; aliquots were removed after various periods of time to determine bound [3H]cAMP by the ammonium sulfate precipitation method (16).

**Scintillation Proximity Assay**—The association of RI with bound [3H]cAMP to immobilized C subunit was determined in buffer A with 0.3 mM ATP or ADP by scintillation proximity assay as described previously (13).

**Separation of the Free and Holoenzyme-associated C Subunit by Size Exclusion Chromatography**—A size exclusion column (Superdex 200, 2.6/30; Amersham Biosciences) was equilibrated with various concentrations of R subunit in buffer A containing one or more of the following substances: 0.3 mM ATP, 0.6 mM ADP, 1.2 mM AMP, 0.03 mM cAMP, 0.1 mM kemptide. The sample (50 μl) contained C subunit and bovine hemoglobin as endogenous standard. Fractions (0.1 ml) were collected and analyzed for kinase activity in the presence of 10 μM cAMP and for absorbance at 405 nm (hemoglobin). The fraction of C complexed with R in a particular sample was determined based on the elution position of the kinase activity in that sample compared with the elution position of free C subunit and the holoenzyme form of the kinase. If the kinase activity eluted midway between the holoenzyme and free C subunit, the sample was considered to contain 50% holoenzyme and 50% free C subunit.

**RESULTS**

Peptide Substrate Competitively Inhibited the Formation of Inactive cAMP-saturated PKA Type I Holoenzyme but Failed to Affect PKA Type II Holoenzyme Formation—In line with our previous report (13), the PKA activity was inhibited at submicromolar concentrations of cAMP-saturated RI or RII subunit. The IC50 for cAMP-saturated RII was 0.8 μM irrespective of the kemptide concentration. On the other hand, several-fold more RI was required to form inactive holoenzyme in the presence of high than low kemptide concentration (Fig. 1, A and B).

To know more about the mechanistic background for this striking difference between the isozymes, we studied the kinase activity at various concentrations of kemptide and at constant concentration of cAMP-saturated R subunit. The RII subunit inhibited the kinase activity in a non-competitive manner (not shown), whereas the RI subunit was a competitive inhibitor of kemptide (Ki = 0.19 μM), as shown by a double inverse plot (Fig. 2). The Ki value was also similar when calculated from experiments where the concentration of RIIC1 was 1 or 3.8 μM (not shown).

To know whether kemptide could also modulate the holoenzyme formation for RI subunit monosaturated with cAMP, we studied the RI(G325D) mutant. This protein did not bind cAMP to site B but retained high affinity cAMP binding to site A (not shown). It could form inactive holoenzyme at subnanomolar concentration in the presence of excess cAMP (Fig. 1C). The concentration of kemptide required to modulate holoenzyme formation was similar for RI(G325D) and RI wild type (Fig. 1). This suggested a competitive relationship between RI and kemptide whether one or both cAMP binding sites of RI were occupied.

**The Physical Formation of PKA Type I and Type II Holoenzyme Complex Was Differentially Modulated by Mg(ADP), Mg/ADP, and Substrate Peptide**—The experiments described above (Figs. 1 and 2) relied on kinase activity as a marker of holoenzyme formation and therefore had to be conducted under conditions allowing substrate phosphorylation by the C subunit of the kinase. Each catalytic cycle of the kinase is associated with

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**Fig. 1.** The inhibition of kinase activity by the cAMP-saturated RI and RII subunits of PKA at various concentrations of kemptide. The C subunit was incubated with 0.1 mM cAMP and various concentrations (abscissa) of RIIC (A), RIIC (B), or RIIC G325D, whose B-site is silent (C). The incubation was for 30 min at 25 °C in buffer A with 0.3 mM [γ-32P]ATP. The concentration of peptide substrate (kemptide) was 7 μM (○), 22 μM (●), 70 μM (▲), or 221 μM (▲). For further details see “Experimental Procedures.” The kinase activity in the absence of added R subunit is taken as unity.

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The remaining trace of C subunit to 2 mM of R subunit with bound [3H]cAMP in buffer A with 0.6 mM ADP or 1.2 mM AMP. Unlabeled CAMP (2 μM) was present to prevent rebinding of the dissociated [3H]cAMP; aliquots were removed after various periods of time to determine bound [3H]cAMP by the ammonium sulfate precipitation method (16).
the formation of C subunit-bound Mg/ADP, whose slow dissociation is the rate-limiting step of the phosphorylation cycle (17). Under phosphorylation conditions the C subunit can therefore exist in complex not only with Mg/ATP or Mg/ATP and kemptide but also with Mg/ADP or Mg/ADP and kemptide or phosphokemptide. One possible explanation for the puzzling difference between PKA types I and II regarding kemptide effect (see above) could be differential affinity of RI and RII subunits for the Mg/ADP-ligated C subunit formed in the presence of kemptide rather than a differential affinity for the kemptide-ligated C subunit itself. To test this possibility we used biophysical methods to determine holoenzyme formation (a) in the presence of Mg/ADP and Mg/ATP in the absence of kemptide, and (b) in the presence of Mg/ADP and absence and presence of kemptide. In this way we could study the effects of Mg/ATP, Mg/ADP, and kemptide without interference from any reaction products formed due to phosphorylation of substrate.

The scintillation proximity assay allows the selective determination of isotope close to the immobilized scintillant. We attached biotinylated C subunit of PKA to the scintillant-containing vial wall and titrated its binding of R with bound \(^{[3H]}\text{cAMP}\) (13) (Fig. 3A). We found that the apparent \(K_d\) for formation of PKA type I holoenzyme (ternary complex of C, RI, and \(^{[3H]}\text{cAMP}\)) rose from 0.16 \(\mu M\) in the presence of Mg/ATP to 1.3 \(\mu M\) in the presence of Mg/ADP (Fig. 3A). The formation of Mg/ADP bound to the C subunit can therefore be one mechanism by which substrate can destabilize the PKA type I holoenzyme.

To know whether kemptide could have a direct destabilizing effect on the RI-C complex, as suggested by the competitive nature of its action (Fig. 2), we studied the formation of PKA holoenzyme in the absence and presence of kemptide. The formation of holoenzyme was determined based on the retention of C subunit injected into a size exclusion chromatography column equilibrated with various concentrations of CAMP-saturated RI subunit. We found that kemptide (100 \(\mu M\)) increased the retention, indicating less holoenzyme formation, whether Mg/ATP (Fig. 3B) or Mg/ADP (Fig. 3C) was present. The effect of kemptide in the presence of Mg/ADP suggests strongly that kemptide can also inhibit holoenzyme formation independently of the conversion of Mg/ATP to Mg/ADP during the phosphorylation cycle.

Experiments similar to those shown for type I holoenzyme were next conducted for type II holoenzyme. We found (Fig. 4A) that the \(K_f\) for formation of holoenzyme complex between CAMP-saturated RII and the C subunit was only slightly higher in the presence of Mg/ADP (1.5 \(\mu M\)) than in the presence of Mg/ATP (0.8 \(\mu M\)). In the presence of Mg/AMP the \(K_f\) of the CAMP-saturated holoenzyme complex was 2.4 \(\mu M\) (Fig. 4A). Kemptide failed to affect the retention of C subunit injected into a column equilibrated with 0.5 \(\mu M\) RII in the presence of Mg/ADP (not shown) or Mg/ATP (Fig. 4B). We conclude that kemptide substrate did not have a direct effect on holoenzyme formation between RII and C subunit, unlike the findings for RI and C subunits. Furthermore, the substrate-dependent formation of Mg/ADP would be far less effective in dissociating PKA type II (Fig. 4A) than type I (Fig. 3A).

**Substrate Peptide Counteracted the C Subunit-induced Dissociation of the RII–\(^{[3H]}\text{cAMP Complex but Not the RII–\(^{[3H]}\text{cAMP Complex}**—A major difference between PKA type I and type II is that only the latter is capable of undergoing autophosphorylation at a residue (Ser-95 in human RII) in the (pseudo)substrate part of the linker region between the N- and C-terminal domains of the R subunit (8–10). The autophosphorylated form of RII is known to bind less tightly to the C subunit (18–20). Kemptide can inhibit the autophosphorylation of RII (21). One can therefore hypothesize that kemptide could facilitate type II kinase dissociation but that this effect is masked by decreased autophosphorylation, leading to little or no effect of substrate on type II kinase dissociation, as observed...
increasing concentration of RII abolished the C subunit-induced dissociation of \([\text{3H}]\text{cAMP}\) from RI (Fig. 5). The dissociation of \([\text{3H}]\text{cAMP}\) from RI was studied in a buffer with Mg/ADP or Mg/ATP in which the phosphorylation state of RII was altered. The degree of autophosphorylation, we conducted experiments (Figs. 1, 3, and 4). To know whether the different response to kemptide for the isozymes was due to any effect of kemptide on the degree of autophosphorylation, we conducted experiments (Fig. 5) in which the phosphorylation state of RII was unaltered.

The C subunit-induced dissociation of labeled cAMP from its complex with RII was studied in a buffer with Mg/ADP or Mg/AMP instead of Mg/ATP to avoid any possibility of RII phosphorylation. We found no effect of kemptide on the rate of the C subunit-induced dissociation of the complex of RII and \([\text{3H}]\text{cAMP}\). This was true for RII incubated with either Mg/ADP (Fig. 5A) or Mg/AMP (Fig. 5B). In contrast, kemptide nearly abolished the C subunit-induced dissociation of \([\text{3H}]\text{cAMP}\) from its complex with RI incubated with Mg/ADP (Fig. 5D) even at 1 \(\mu\)M C subunit (Fig. 5E).

To study phospho-RII, we incubated with Mg/AMP because the C subunit can catalyze dephosphorylation of phospho-RII in the presence of Mg/ADP (22) or in the presence of Mg/ATP and kemptide (21). We found no effect of kemptide on the rate of the C subunit-induced dissociation of the complex of phosphorylated RII and \([\text{3H}]\text{cAMP}\) (Fig. 5C). This shows that kemptide acted differently on the association of C to RI and RII also under conditions when autophosphorylation of RII or dephosphorylation of phospho-RII could be ruled out.

An inhibitor peptide, representing the 20-residue core of the heat-stable inhibitor of PKA (14) binds to the substrate peptide binding site as well as to an adjacent hydrophobic area on the C subunit (23). Mutagenesis of residues in this area of the C subunit inhibited the interaction with RII (11). It was therefore of interest from a mechanistic point of view to know whether the peptide could modulate the interaction of C subunit and the complex of RII and \([\text{3H}]\text{cAMP}\). This peptide at 1 \(\mu\)M inhibited the dissociation of \([\text{3H}]\text{cAMP}\) from RI (Fig. 5D) and RII. Even at 50 nM it was able to counteract the C subunit-induced dissociation of cAMP from RII (Fig. 5A). This showed that the assay was able to pick up effects of peptides competing with RII for binding to the C subunit.

**Substrate Sensitized PKA Type I, but Not Type II, toward Activation by cAMP**—To know whether peptide substrate could sensitize PKA toward cAMP, we studied the cAMP-dependence of PKA types I and II in the presence of various concentrations of kemptide. Whereas 0.074 \(\mu\)M cAMP was sufficient to half-maximally activate PKA type I at near saturating concentration of substrate peptide, 0.21 \(\mu\)M cAMP was required at low peptide concentration (Fig. 6A). Little sensitization by substrate was noted for PKA type II, which was 50% activated at 0.4–0.5 \(\mu\)M cAMP (Fig. 6B).

A concern was that kemptide failed to sensitize the type II isozyme because of its small physical size and that a macromolecular substrate would also sensitize PKA type II. To test this possibility we used as substrate a macromolecule where a PKA substrate consensus sequence (SRRASGL) was incorporated as a linker between the two macromolecules glutathione S-transferase and 14–3-3\(\gamma\). We also studied the physiological and large substrate tyrosine hydroxylase, which exists as a tetramer of four subunits of 60 kDa (24). Tyrosine hydroxylase is a physiological substrate whose activity is regulated by phosphorylation by PKA (25). We found that either of these macromolecular substrates, like kemptide, sensitized PKA type I, but not type II, toward activation by cAMP (Fig. 7).
that physiologically relevant macromolecular PKA substrates also facilitate cAMP activation of PKA type I, but not type II.

**DISCUSSION**

The biological significance of the existence of two major isozymes (PKA types I and II) of the cAMP-dependent protein kinase with distinct regulatory subunits (RI, RII) is still enigmatic. The amino acid sequence of the RI and RII subunits differs most in the N-terminal region, which interacts with the A kinase-anchoring protein family of scaffolding proteins. It is therefore likely that one teleological purpose of the existence of two isozymes is to obtain differential subcellular localization of the kinase depending on which isozyme is expressed.

The present study demonstrates another striking difference. The presence of substrate peptide sensitized PKA type I to the activating effect of cAMP. This was true whether the substrate was a minimal heptapeptide or a macromolecular substrate like tyrosine hydroxylase (Figs. 6 and 7). This means that for PKA type I the degree of activation by a moderate cAMP increase would depend on the concentration of available substrate in the immediate vicinity. We believe that such substrate activation is a new way in which PKA type I can be activated without altering the cAMP level. The substrate histone was already reported in 1973 to induce dissociation and sensitize PKA type II toward cAMP (26). We found little sensitizing effect of substrate toward type II kinase in the present study, using a number of substrates. Presumably, the effect of histone can be explained by nonspecific electrostatic interaction with RII not related to its ability to act as substrate (27).

PKA type II is anchored close to ion channels that are PKA substrates (28, 29). For the L-type Ca\(^{2+}\) channels in heart there is a requirement for rapid activation and inactivation during the cardiac contraction cycle (reviewed in Refs. 30 and 31). A functional requirement for rapid kinase inactivation following an abrupt decrease of [cAMP] may also be present for other channels regulated by anchored PKA type II (7). The rate with which PKA is reactivated after a burst of cAMP depends on the rate of recombination between the C subunit and the CAMP-saturated R subunit. Each phosphorylation cycle generates C(Mg/ADP), which is believed to be more abundant than C(Mg/ATP) under steady state conditions of active substrate phosphorylation, because each catalytic cycle of the kinase is associated with the formation of C subunit-bound Mg/ADP, whose slow dissociation is the rate-limiting step of the phosphorylation cycle (17, 21, 32). We found that C(Mg/ADP) reassociated ~15-fold more rapidly with CAMP-saturated RI\(\alpha\) than RII\(\alpha\). In the presence of kemptide the difference was even larger (Fig. 5). It appears therefore that a short-lived burst of cAMP is likely to lead to a prolonged activation (feed-forward reaction) for PKA type I, but not type II. This may provide a functional explanation for why PKA type II is preferred in complexes with substrates undergoing rapid phosphorylation cycles.

The cAMP responsiveness of PKA type I in the intact cell is enigmatic. There is a general notion that PKA type I is more responsive than PKA type II to slight increases of cAMP (33, 34). The higher cAMP sensitivity of PKA type I than type II in the presence of abundant substrate (Figs. 6 and 7) can explain the preferential activation of PKA type I by slight cAMP stimulation. On the other hand, RI\(\alpha\) is known as the “tissue-specific extinguisher” of cAMP-induced gene activation (35, 36), implying that it can counteract cAMP-dependent transcription more...
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makes PKA type II more suitable for a rapidly reversible activation after a burst of cAMP. This may provide a functional explanation for why PKA type II is preferred in compartmentalized complexes with substrates undergoing rapid phosphorelay cycles.

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