Exogenous substrate stimulates autodephosphorylation of cyclic-AMP-dependent protein kinase II

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The autophosphorylated regulatory subunit (^{32}P -RII) of cyclic-AMP-dependent protein kinase II was efficiently dephosphorylated by its C subunit in the absence of added ADP, provided that Mg/ATP and a standard protein kinase peptide substrate were present. This raises the possibility that autodephosphorylation could be significant in the intact cell. Only the cyclic-AMP-complexed free form of ^{32}P -RII was efficiently dephosphorylated, indicating that the autodephosphorylation was intermolecular. Autodephosphorylation of ^{32}P -RII in the

presence of MgATP and kemptide occurred with formation of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$, suggesting transfer of ^{32}P of phospho-RII to a transient $\text{C}^*(\text{MgADP})$ complex formed during the forward kinase reaction with peptide as substrate. Autodephosphorylation promoted by phosphorylation of exogenous substrates could operate also for other kinases conforming to a mechanism where MgADP remains bound to the active site after the other product (phosphorylated substrate) has left the catalytic complex.

INTRODUCTION

Enzymes can in principle catalyse a reaction in both the forward and the reverse direction. This is also the case for the cyclic-AMP (cAMP)-dependent protein kinase (cA-PK), which catalyses the reversible reaction:

This enzyme exists in two main isoenzyme forms, cA-PKI and cA-PKII, and has a pivotal role in mediating the effects of the second messenger cAMP by phosphorylation of key cell proteins. The type II isoenzyme undergoes an autophosphorylation reaction, where a specific serine residue in the 'hinge' region of the regulatory moiety (RII) is phosphorylated by the catalytic (C) moiety. For general reviews see Beebe and Corbin (1986), Edelman et al. (1987), Taylor et al. (1990), McKnight (1991) and Døskeland et al. (1993).

The protein-kinase-catalysed reaction (eqn. 1) is considered virtually irreversible within the cell, because the forward reaction is faster than the reverse reaction (Shizuta et al., 1975; Rosen and Erlichmann, 1975; Døskeland et al., 1984), and because of the low proportion of intracellular MgADP relative to MgATP.

The present study reports the novel finding that, for autophosphorylated RII, reversal of the kinase reaction did not require added ADP, provided that the C subunit was supplied with standard peptide substrate and MgATP. This raises the possibility that the reaction can be of significance in the intact cell, i.e. even when MgATP predominates relative to MgADP. A mechanism is proposed for the coupling between autodephosphorylation of P-RII and phosphorylation of peptide substrate, involving a transient C-MgADP complex formed during C-catalysed phosphorylation of standard peptide substrates.

EXPERIMENTAL

Materials

$[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and $[5',8\text{-}^3\text{H}]\text{cAMP}$ were from The Radiochemical Centre, Amersham, Bucks., U.K. ATP-regenerating system (phosphocreatine and creatine kinase) was from Boehringer, Mannheim, Germany. Microcystin-LR, phosphate-acceptor heptapeptide (Leu-Arg-Arg-Ala-Ser-Leu-Gly, 'kemptide') and all unlabelled nucleotides were from Sigma, St. Louis, MO, U.S.A. Reagents for the Bradford (1976) protein assay and a mixed globulin protein standard were obtained from Bio-Rad, Richmond, CA, U.S.A. A synthetic peptide corresponding to residues 5–24 of the heat-stable cA-PK inhibitor protein (Scott et al., 1986) was generously given by Dr. J. Scott and Dr. E. G. Krebs, University of Washington, Seattle, WA, U.S.A. C subunit and cA-PKII were prepared from bovine myocardium, essentially as described by Sugden et al. (1976) and Øgreid and Døskeland (1980), respectively.

Determination of $[\text{H}]\text{cAMP}$ binding to R; assay for kemptide

Determination of $[\text{H}]\text{cAMP}$ binding to RI or RII was based on measurement of their binding capacity for $[\text{H}]\text{cAMP}$ and separation by using specific antibodies (Ekanger et al., 1989). The C-subunit concentration was estimated by mixing various concentrations with purified RI of known concentration (in the absence of cAMP) and titrating the resulting free kinase activity by assaying its phosphotransferase activity at 30 °C in 50 mM potassium phosphate/15 mM Hepes/10 mM magnesium acetate/70 μM phospho-acceptor peptide (kemptide)/0.1 mM $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (2 mCi/mmol) / 0.5 mM EGTA / 1 mM dithioerythritol. This estimate was 15 % lower than the estimate based on protein content and $M_r = 40000$, suggesting either that the

Abbreviations used: cAMP, cyclic AMP; cA-PKI, cA-PKII, cAMP-dependent protein kinase type I and type II respectively; RI, RII, the regulatory subunits of cA-PKI and cA-PKII respectively; C, the catalytic subunit of cA-PKI or cA-PKII; k_{de} , rate constant for pseudo-first-order dephosphorylation; K_d , apparent equilibrium dissociation constant; C(MgADP), C subunit with MgADP bound to its active site as a result of direct binding of MgADP; $\text{C}^*(\text{MgADP})$, C subunit with bound MgADP formed *in situ* from bound MgATP upon peptide phosphorylation; kemptide, the phosphate-acceptor heptapeptide Leu-Arg-Arg-Ala-Ser-Leu-Gly.

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Table 1 Test of the ability of selected substances to modulate the C-subunit-catalysed dephosphorylation of ^{32}P -RII

Pre-autophosphorylated ^{32}P -cA-PKII was subjected to dephosphorylation in the presence of the C subunit of cA-PK and the agents indicated in the first column of the Table. Other conditions were as described in the Experimental section. The initial rate of dephosphorylation was determined and is expressed relative to the rate observed in the presence of MgATP and kemptide. The apparent K_m value for MgADP and the K_i value of MgATP (with 30 μM or 100 μM ADP) are also shown.

Substance tested [Concentration(s)]	Dephosphorylation rate (relative to that with MgATP/kemptide)	Range (n)
5 mM Mg + 1 μM $\rightarrow$ 3 mM ADP	0.07 $\rightarrow$ 0.79 ($K_m = 11$ μM)	K_m 8–14 ($n = 3$)
5 mM MgADP + 1 μM $\rightarrow$ 4 mM ATP	0.78 $\rightarrow$ 0.04 ($K_i = 14$ μM)	K_i 11–19 ($n = 3$)
5 mM Mg + 1 mM GDP	0.02	0.00–0.04 ($n = 2$)
5 mM Mg + 3 mM ATP + 0.3 mM kemptide	1.0	–
1.5 μM H-8 + MgATP/kemptide	0.42	0.38–0.45 ($n = 2$)
100 μM H-8 + MgATP/kemptide	0.07	0.05–0.08 ($n = 2$)
10 μM KT-5720 + MgATP/kemptide	0.19	0.17–0.21 ($n = 2$)
1 mg/ml Polylysine + MgATP/kemptide	0.04	0.03–0.05 ($n = 2$)
1 μM cA-PK inhibitor peptide + MgATP/kemptide	0.14	0.11–0.17 ($n = 2$)
10 μM Microcystin-LR + MgATP/kemptide	1.0	0.95–1.06 ($n = 2$)

protein standard was not accurate for the C subunit when the Bradford (1976) assay was used, or that some of the C-subunit protein was catalytically inactive.

The amount of phospho-acceptor peptide (kemptide) present in a sample was estimated by allowing the phosphorylation to proceed to apparent completion. The assay was at 30 °C in 50 mM potassium phosphate / 15 mM Hepes / 10 mM magnesium acetate / 0.3 mM $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (1 mCi/mmol) / 0.5 mM EGTA / 0.1 mM EDTA / 1 mM dithioerythritol / 100 nM C subunit. Samples were removed after 20, 30 and 40 min of incubation. Completeness of substrate phosphorylation was indicated by a plateau of incorporation as a function of incubation time.

Assay for autodephosphorylation of cA-PKII

The cA-PKII holoenzyme was routinely autophosphorylated by preincubation for 15 min at 0 °C in 15 mM Hepes/NaOH, pH 7.2, containing 20 mM KCl, 1 mM EDTA, 0.3 mM EGTA, 0.3 mg/ml BSA and 0.15 mg/ml of soybean trypsin inhibitor, with 2.5 mM magnesium acetate and 2 μM $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (60 Ci/mmol) in a volume of 0.25 ml. Excess radioisotope was removed by passing the preincubation mixture through a Sephadex G-25 column (1.2 ml bed volume) equilibrated with 50 mM potassium phosphate (pH 7.2) / 10 mM sodium pyrophosphate / 20 mM NaF / 5 mM EDTA / 5 mM EGTA. Fractions (60 μl) were collected, and the three first flow-through fractions were pooled. The amount of ^{32}P -RII recovered was the same whether or not 2 μM microcystin was included during preincubation and in the column-equilibration buffer.

The dephosphorylation reaction was started by adding 20 μl of the pooled ^{32}P -labelled cA-PKII fractions to 1.3 ml of buffer (15 mM Hepes/NaOH, pH 7.2, 20 mM KCl, 1 mM EDTA, 0.3 mM EGTA, 0.3 mg/ml of BSA, 0.15 mg/ml of soybean trypsin inhibitor) containing routinely 6 mM magnesium acetate, 30 μM cAMP and 50–100 nM of the catalytic subunit of cA-PK. Various concentrations of ADP, ATP, ATP-regenerating system (phosphocreatine and creatine kinase) and other agents to be tested (Table 1) were present in some incubations. The dephosphorylation reaction was arrested by spotting 150 μl samples on to filter-paper discs (25 mm diameter), which were immediately transferred to 10% (w/v) trichloroacetic acid with 5 mM pyrophosphate. After washing (10 ml/filter) six times in 5% trichloroacetic acid, the filters were treated sequentially with

ethanol and acetone, dried, and counted for radioactivity in a liquid-scintillation counter.

Determination of pseudo-first-order rate constants for dephosphorylation of ^{32}P -RII: estimation of the fractional occupancy of the binding site governing the cAMP effect on autodephosphorylation

When the initial rate of dephosphorylation of ^{32}P -RII obeyed apparent first-order kinetics, the operational rate constant (k_{de}) for the dephosphorylation process was calculated simply from the relation:

$$k_{\text{de}} = \ln 2 / t_{\frac{1}{2}}$$

where $t_{\frac{1}{2}}$ is the time (in seconds) required for 50% dephosphorylation of the phosphoprotein initially present.

Assuming that cAMP accelerated the dephosphorylation of P-RII by binding to a site, and acted proportionally with the fractional occupancy of this site, it follows that:

Fractional occupancy =

$$[k_{\text{de}}(x) - k_{\text{de}}(0)] / [k_{\text{de}}(\text{max. cA}) - k_{\text{de}}(0)]$$

where $k_{\text{de}}(0)$ is the rate constant for dephosphorylation in the absence of added cAMP, $k_{\text{de}}(x)$ that in the presence of concentration x of cAMP, and $k_{\text{de}}(\text{max. cA})$ that at maximally effective cAMP concentration. A similar approach has been used to estimate the occupancy of one binding site governing the behaviour of another binding site (Døskeland et al., 1987).

Determination of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ produced during the autodephosphorylation of ^{32}P -RII

Pre-autophosphorylation was as described above, except that the concentration of cA-PKII was increased 5-fold and the specific radioactivity of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ was increased 2-fold. $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ was removed from the preincubation by passing it twice through Sephadex G-25 columns, each twice the routine size. Since the desalted preincubation was diluted only 3-fold in the dephosphorylation reaction, the second column was equilibrated with only 5 mM NaF, 5 mM pyrophosphate and 2 mM each of EGTA and EDTA, to avoid excessive chelation of Mg^{2+} during the ensuing autodephosphorylation reaction. Less than 2% of the ^{32}P in the sample subjected to autodephosphorylation was soluble in 0.6 M HClO_4 .

Autodephosphorylation was for 7.5 and 15 min with 150 nM

C subunit, 300 μ M kemptide and 0.5 mM ATP. The ATP-regenerating system and other ingredients were as described above. The reaction was also performed in the absence of kemptide, ATP or ATP-regenerating system, both with and without 3 mM ADP. In one series of experiments the autodephosphorylation was compared between the incubations containing MgADP + complete ATP-regenerating system and those containing MgADP with phosphocreatine and boiled creatine kinase. The reactions were arrested by addition of HClO_4 and separated by h.p.l.c. essentially as described by Døskeland et al. (1992), by using a LiChrosorb RP-18 UltraPac column (4 mm $\times$ 250 mm) with 2% (v/v) methanol, 110 mM potassium phosphate (pH 6.9) and 0.3 mM tetrabutylammonium hydrogen sulphate as the mobile phase. Fractions of volume 0.3 ml were collected and assayed for radioactivity.

To test whether the labelled substance co-chromatographing with authentic ATP was $[\gamma\text{-}^{32}\text{P}]\text{ATP}$, the peak h.p.l.c. fractions of presumed $[\text{P}^{32}]\text{ATP}$ were tested for ability to transfer ^{32}P to peptide substrate under standard protein kinase assay conditions with 0.1 mM ATP, 150 nM C subunit and 300 μ M kemptide. The reaction was allowed to run to apparent completion, as ascertained by a plateau of incorporation of ^{32}P into substrate (achieved after less than 20 min of incubation). The degree of incorporation of ^{32}P from $[\text{P}^{32}]\text{ATP}$ produced in the autodephosphorylation reaction was compared with the incorporation using as phosphate donor $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ freshly obtained from the producer, and diluted in h.p.l.c. mobile phase.

Autoradiography of cA-PKII autophosphorylated in the absence and presence of kemptide and cAMP

cA-PKII holoenzyme (0.1 μ M with respect to subunit concentration) was incubated at 0 $^{\circ}\text{C}$ in 15 mM Hepes/NaOH, pH 7.2, 20 mM KCl, 1 mM EDTA, 0.3 mM EGTA, 0.3 mg/ml BSA, 0.15 mg/ml soybean trypsin inhibitor, 2.5 mM magnesium acetate and 4 μ M $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (60 Ci/mmol). In some incubations cAMP (30 μ M) or substrate peptide (300 μ M) was present. The incubation volume was 100 μ l. Samples of volume 20 μ l were removed, mixed first with 0.5 ml of 10% trichloroacetic acid, and then with 5 μ g each of BSA, ovalbumin and soybean trypsin inhibitor. The precipitate was collected by centrifugation, extracted with diethyl ether, and dissolved in 40 μ l of 10 mM Tris/phosphate, pH 8, with 9 M urea, 2 mM dithioerythritol and 1 mM ethylamine. This sample was diluted 1:1 in sample buffer and subjected to SDS/PAGE (11% total acrylamide concentration) as described by Laemmli (1970). The washed and subsequently dried gel was exposed to radiographic film (β -MAX from Amersham).

RESULTS

Introductory experiments

The RII subunit of cA-PKII showed an anomalous decrease in its apparent cAMP affinity as a function of incubation time in tissue extracts supplemented with MgATP. It was considered possible that this was due to loss of phosphate from the autophosphorylation site of RII. However, neither inhibitors directed against phosphatases 1 and 2A (microcystin) or the Ca^{2+} /calmodulin-dependent phosphatase 2B (EGTA, W-7 and trifluoperazine) nor NaF prevented the decrease of cAMP binding to RII [the nomenclature and inhibitor-sensitivity of protein phosphatases has been reviewed by Cohen (1989) and Bollen and Stalmans (1992)]. In a further attempt to inhibit phosphatases, the cA-PK substrate kemptide (500 μ M) was added to achieve an increasing concentration of the presumed

phosphatase inhibitor phosphokemptide. Surprisingly, this further decreased the cAMP binding to RII. Decreased apparent cAMP affinity was also noted when kemptide was presented to purified cA-PKII incubated with MgATP. These findings led us to suspect that kemptide and MgATP enhanced the dephosphorylation of P-RII independently of phosphatases. This possibility was studied by using isolated cA-PKII.

The C subunit of cA-PK catalyses dephosphorylation of P-RII in the presence of saturating MgATP and substrate peptide

Autophosphorylated $^{32}\text{P}\text{-RII}(\text{cAMP})_2$ was indeed rapidly dephosphorylated in the presence of kemptide, MgATP and the C subunit of cA-PK (Figure 1). The initial reaction obeyed apparent first-order kinetics (Figure 1a), and the first-order rate constant for dephosphorylation was similar for all $^{32}\text{P}\text{-RII}(\text{cAMP})_2$ concentrations (1–50 nM) tested (results not shown), as expected for a reaction in which the substrate concentration is far below its K_m value. The reaction was not supported by phosphokemptide (Figure 1a), and required Mg^{2+} , and was dependent on the C-subunit concentration (Figure 1b). The effect of the C

Figure 1 Dephosphorylation of $^{32}\text{P}\text{-RII}$ is catalysed by the C subunit of cA-PK in the presence of MgATP and kemptide

Panel (a) shows the dephosphorylation of $^{32}\text{P}\text{-RII}$ by C subunit (60 nM) in the presence of kemptide (0.3 mM), with (■) or without (●) an ATP-regenerating system (4 mM phosphocreatine + 10 units/ml creatine kinase; Cr-P/CPK). With 50 μ M kemptide (△) the dephosphorylation rapidly abated. Phosphokemptide (◇) failed to support rapid dephosphorylation. Panel (b) shows the Mg^{2+} requirement and the C-subunit concentration-dependence of the reaction. The cA-PKII holoenzyme was pre-autophosphorylated as described in the Experimental section, and 20 μ l (containing 2.5 pmol of $^{32}\text{P}\text{-RII}$) was exposed to dephosphorylation by mixing with 1.3 ml of buffer (15 mM Hepes/NaOH pH 7.2, 20 mM KCl, 1 mM EDTA, 0.3 mM EGTA, 0.3 mg/ml BSA, 0.15 mg/ml soybean trypsin inhibitor) containing 10 mM magnesium acetate, 3 mM ATP and 30 μ M cAMP. In one experiment (□) magnesium acetate was omitted. For the experiments shown in (b) the buffer contained 30 nM (▲), 90 nM (■) or no (○) added C subunit. About 2 nM C subunit was carried over with the preincubation mixture. Samples (150 μ l) were spotted on to filter-paper discs to determine $^{32}\text{P}\text{-RII}$, as described in the Experimental section.

subunit depended on its concentration, rather than the ratio between P-RII and C subunit concentrations (tested from 1:1 to 1:200), indicating a catalytic effect of the C subunit, as expected when excess cAMP is present to prevent 'trapping' of C subunit in cA-PKII holoenzyme complex.

The dephosphorylation was dependent on continuous phosphorylation of kemptide, since the reaction rate declined as the amount of kemptide became insufficient to support the kinase activity (Figure 1a). This was demonstrated by starting the reaction with a concentration of kemptide (50 μ M) that, at the level of C subunit present, became nearly completely converted into phospho-kemptide during the reaction period. The conversion of kemptide into phospho-kemptide was determined in a separate experiment run under similar conditions to those shown in Figure 1(a) with 50 μ M kemptide and using cA-PKII holoenzyme preincubated without labelled ATP. Samples were diluted 4-fold in a protein kinase assay with 200 μ M [γ - ^{32} P]ATP and 100 nM C subunit for 15 min, and ^{32} P-kemptide was determined. Only 20 % of the originally added 50 μ M kemptide was still phosphorylatable after 30 s of incubation, and less than 5 % was left after 60 s (results not shown).

The need for catalytic activity of the C subunit was supported by the lower rate of autodephosphorylation in the presence of kinase inhibitors (Table 1), such as a peptide corresponding to the active sequence of the heat-stable protein kinase inhibitor, and the ATP-directed inhibitors H-8 and KT-5720 (reviewed by Hidaka and Kobayashi, 1992). The abolition of autodephosphorylation by polylysine (Table 1) was presumably due to blocking of the interaction between RII and the C subunit, since polylysine prevented the reassociation of RII and C subunit (results not shown).

Evidence that MgADP, formed *in situ* on the C subunit from MgATP, is the phosphate acceptor during autodephosphorylation of cA-PKII

The molecule accepting the ^{32} P from ^{32} P-RII(cAMP) $_2$ during autodephosphorylation in the presence of kemptide, MgATP and an ATP-regenerating system was sought by h.p.l.c. analysis of the HClO $_4$ -soluble fraction from such an incubation. More than 90 % of the HClO $_4$ -soluble ^{32} P was identified as [^{32} P]ATP. About 75 % of this [^{32} P]ATP could be incorporated into kemptide under protein kinase assay conditions, where 70–75 % of authentic [γ - ^{32} P]ATP was incorporated. [γ - ^{32} P]ATP was therefore the main, and possibly the only, radioactive product formed from ^{32} P-RII during autodephosphorylation. The origin of the ADP required to accept the labelled phosphate from ^{32} P-RII to produce [γ - ^{32} P]ATP was not immediately evident, since no exogenous ADP had been added to the incubation. Furthermore, an efficient (see below and Figure 2) ATP-regenerating system was present, and any free ADP molecules would have had to compete (Table 1, Figure 2) with a vast excess of ATP in the reaction mixture. The acceptor ADP therefore had to be present at a site inaccessible to the ATP-regenerating system and not in equilibrium with the general ATP pool in the incubation.

The experiments shown in Figure 2 were performed to find to what extent MgADP, added to the reaction medium, could substitute for the ADP produced *in situ* (see above). One would expect that only a fraction ($[C \cdot (MgADP)]/[total\ C]$) of the C-subunit molecules carried MgADP during kemptide phosphorylation in the presence of MgATP (see right-hand side of Table 2). Therefore, depending on the size of this fraction, the autodephosphorylation with MgATP and kemptide should be slower than in the presence of millimolar MgADP, which should nearly saturate the C subunit with MgADP. Surprisingly, when

Figure 2 MgADP, but not MgATP, can promote autodephosphorylation in the absence of kemptide

The time course of ^{32} P-RII dephosphorylation was studied in the presence of 10 mM Mg $^{2+}$, 30 μ M cAMP and 75 nM C subunit. The rate of dephosphorylation was low without added nucleotide (○) as well as with 3 mM ATP (□). With 3 mM ADP (▲) alone the dephosphorylation was about twice as fast as when equimolar (3 mM ADP/3 mM ATP) amounts of ADP and ATP (■) were present. The effect of ADP (0.3 mM) was efficiently counteracted by 3 mM ATP (△), and even the effect of 3 mM ADP was abolished (●) by an ATP-regenerating system (Cr-P/CPK, as in Figure 1).

the dephosphorylation rate was compared in incubations run in parallel with MgADP and with MgATP-kemptide, it was slightly (20 %), but significantly, faster under the latter conditions (Table 1). The effect of ADP (kemptide absent) was completely blocked by an ATP-regenerating system (phosphocreatine/creatine kinase), and was competitively inhibited by ATP (Figure 2, Table 1).

The apparent K_m (11 μ M) for MgADP in the autodephosphorylation reaction (Table 1) was close to the K_i of MgADP estimated from the ability of MgADP to inhibit competitively cA-PK-catalysed phosphorylation of substrates (Whitehouse et al., 1983; results not shown). A low K_m for MgADP was also noted in the early study of reversal of ^{32} P-RII phosphorylation by Rosen and Erlichmann (1975). On the other hand, a K_m value for MgADP of 3.3 mM was found for the reversal of ^{32} P-casein phosphorylation by the C subunit in an important early study (Shizuta et al., 1975). This raises the interesting possibility that dephosphorylation of standard phosphorylated substrates, unlike autodephosphorylation, has a MgADP-dependent step (possibly a ternary complex between C, P-casein and MgADP), with a low affinity for MgADP. An alternative explanation is that the high ^{32} P-casein concentration used (four orders of magnitude higher than that of ^{32} P-RII in the present study) may have led to the accumulation of enough [γ - ^{32} P]ATP to give significant rephosphorylation of substrate at moderate concentrations of ADP. A very recent study of C-subunit-catalysed dephosphorylation of P-kemptide reported apparent K_m values for MgADP in the μ M range (Quamar et al., 1992).

The autodephosphorylated RII could be rephosphorylated with retained mobility on SDS/PAGE, and retained its [^{3} H]cAMP-binding characteristics, indicating that the loss of ^{32} P from the autophosphorylation site was not caused by or accompanied by any degradation or denaturation of RII.

Effect of cAMP and kemptide on the autophosphorylation and autodephosphorylation of cA-PKII

The dephosphorylation of ^{32} P-RII catalysed by the C subunit in

Table 2 Scheme explaining preferential dephosphorylation of P-RII in the presence of kinase peptide substrate

The scheme shows the possible complexes involving C subunit, MgADP or MgATP, RII(cA)₂ and P-RII(cA)₂ in the absence (left; solid arrows) and the presence (right; broken arrows) of exogenous peptide substrate [which, unlike RII(cA)₂, is present at a concentration well above its *K_m* value for phosphorylation]. In the absence of exogenous substrate RII(cA)₂ is readily phosphorylated. In the presence of substrate, dephosphorylation of P-RII(cA)₂ prevails relative to phosphorylation of RII(cA)₂, because: (1) peptide substrate occupies C(MgATP), which is thereby made unavailable for phosphorylation of RII(cA)₂; (2) peptide phosphorylation generates C*(MgADP), which catalyses dephosphorylation of P-RII(cA)₂. The concentration of ATP is taken to be high, and well above that of ADP (as in the intact cell). Compounds of low abundance are shown in *italics* and others in **bold**. Further explanations are given in the text.

Figure 3 cAMP-dependence of the cA-PKII autodephosphorylation reaction

The time course of ³²P-RII dephosphorylation was studied under the conditions described in the legend to Figure 1 (10 mM Mg²⁺, 2 mM ADP and 60 nM C subunit were present in all incubations). The dephosphorylation was strongly dependent on the concentration of cAMP in the medium, which was 0 (○), 0.1 μM (△), 1 μM (□), 10 μM (●), or 0.1 mM (▲).

the presence of added MgADP was enhanced several-fold by cAMP (Figure 3). The simplest explanation is that only free ³²P-RII(cAMP)₂ (not ³²P-RII in the cA-PKII holoenzyme) was prone to significant dephosphorylation. This was supported by the closely corresponding cAMP-saturation curves (Figure 4) based on data calculated from the concentration-dependence of the cAMP effect on autodephosphorylation and actual [³H]cAMP-binding data (determined in parallel assays). In another set of experiments isolated cA-PKII holoenzyme was incubated with Mg[γ-³²P]ATP in the absence and presence of

Figure 4 Correlation between cAMP binding and cAMP promotion of autodephosphorylation

The rate constant for the pseudo-first-order autodephosphorylation reaction was calculated from data (obtained with different cAMP concentrations) as shown in Figure 3, and the fractional occupancy of the putative cAMP binding site(s) responsible for the cAMP effect was calculated (see the Experimental section for details) and plotted (○) as a function of the cAMP concentration during autodephosphorylation. The Figure also shows data for [³H]cAMP binding to RII (●) under similar conditions (presence of MgADP, 60 nM C subunit, 37 °C) to those used for the autodephosphorylation experiment. Note the close correlation between the increase in the two parameters as a function of rising concentrations (abscissa) of cAMP (labelled or unlabelled).

Figure 5 Kemptide blocks the intermolecular, but not the intra-holoenzyme, autophosphorylation of cA-PKII

cA-PKII holoenzyme (0.1 μ M with respect to subunit concentration) was incubated at 0 °C for 20 s (lanes 1, 3, 5, 7) or 60 s (lanes 2, 4, 6, 8) in the buffer described in the legend to Figure 1, but with 2.5 mM Mg^{2+} and 4 μ M [γ - ^{32}P]ATP. In some incubations (lanes 3, 4 and 7, 8) cAMP (30 μ M) was present. In others (1–4) 300 μ M of the kinase substrate kemptide was present. The autoradiographic intensity of the RII bands show that kemptide can block the intermolecular phosphorylation of RII (lanes 3, 4), but not the intramolecular phosphorylation occurring in the absence of dissociating cAMP (lanes 1, 2). The incubation volume was 100 μ L, and 20 μ L samples were removed for SDS/PAGE and autoradiography as detailed in the Experimental section. The arrowheads at the left show the positions (determined from the protein staining pattern) of standard proteins (BSA and ovalbumin respectively) of M_r 67 000 (67 k) and 44 000 (44 k).

cAMP. In either case a rapid phosphorylation of RII was observed in the absence of kemptide. Kemptide selectively prevented phosphorylation of RII in the presence of cAMP (Figure 5), i.e. when RII was dissociated from the cA-PKII holoenzyme. It appeared therefore that kinase substrates such as kemptide counteract the accumulation of autophosphorylated RII by both inhibiting phosphorylation (Figure 5; Table 2) and promoting dephosphorylation (Figure 1; Table 2).

DISCUSSION

Coupling of autodephosphorylation of P-RII and phosphorylation of standard peptide substrate: implications for the kinetic mechanism of the protein kinase reaction

The present study has shown that autodephosphorylation of ^{32}P -RII(cAMP)₂ can be coupled to phosphorylation of a standard protein kinase substrate with the production of [γ - ^{32}P]ATP. This implies that MgADP produced *in situ* on the C subunit of cA-PK can accept directly the ^{32}P from the autophosphorylated site on RII, i.e. that the kinase reaction can proceed from the ternary catalytic complex [C(MgATP)peptide] according to:

Furthermore, the intermediary C*(MgADP) must be able to react with P-RII according to:

[that only one reaction direction is shown does not imply

irreversibility; C*(MgADP) denotes C subunit with bound MgADP formed *in situ* through transfer of phosphate from MgATP to peptide substrate].

Several detailed studies (containing references to important earlier studies on the subject) deal with the kinetic mechanism of the kinase reaction of catalysed by cA-PK (Whitehouse et al., 1983; Kong and Cook, 1988; Quamar et al., 1992) and the multifunctional Ca^{2+} /calmodulin-dependent protein kinase II (Kwiatkowski et al., 1990; Katoh and Fujisawa, 1991a). Data have been presented supporting sequential binding of MgATP and peptide, followed by ordered release of P-peptide and MgADP (Whitehouse et al., 1983; Kwiatkowski et al., 1990) as well as random binding (Kong and Cook, 1988; Katoh and Fujisawa, 1991a; Quamar et al., 1992). The present study probably provides the most explicit evidence so far for release of phosphopeptide before MgADP. Furthermore, the C*(MgADP) complex so formed is able to bind and catalyse dephosphorylation of P-RII(cAMP)₂. This means that the kinase reaction can also be reversed in an ordered manner, with P-RII(cAMP)₂ joining the preformed C*(MgADP).

In apparent contradiction to this, a careful isotope-partitioning study failed to detect phosphotransfer from phosphokemptide to C(MgADP), indicating that ADP dissociated faster than P-kemptide from the complex C(MgADP)P-peptide (Kong and Cook, 1988). This discrepancy can be resolved by assuming that C*(MgADP) differs from C(MgADP) by promoting more efficiently the reversal of the kinase reaction. This idea receives some support from the present study (Table 1): in the presence of MgATP and kemptide [when C*(MgADP) is formed] the autodephosphorylation was slightly faster than in the presence of MgADP [when C(MgADP) dominates]. Unfortunately, the prevalence of C*(MgADP) relative to other complexes of the C subunit (Table 2) is unknown. If 10% of total C subunit is in the C*(MgADP) state in the presence of MgATP and kemptide, C*(MgADP) is about 12-fold more efficient than C(MgADP) in catalysing P-RII(cAMP)₂ dephosphorylation. If 90% is in the C*(MgADP) state, the difference in catalytic activity must be slight.

Whereas the autophosphorylation of RII can proceed as both an intramolecular and an intermolecular reaction (Rangel-Aldao and Rosen, 1976; Figure 5), autodephosphorylation was detected only for P-RII complexed with cAMP (Figures 3 and 4), implying that it occurred only as an intermolecular reaction.

Possible physiological significance of autodephosphorylation of RII

In standard low-salt buffer, 50% dephosphorylation of ^{32}P -RII(cAMP)₂ was achieved in about 30 s by 90 nM C subunit (Figure 2). The concentration of C subunit (0.78 μ mol/kg) in brain (Hofmann et al., 1977) translates to about 1.6 μ M in the cell water phase, assuming that the latter occupies 50% of the brain volume. Extrapolation would indicate an autodephosphorylation rate of 0.4 s⁻¹. This compares favourably with the value (0.45 s⁻¹) recently estimated from data on phosphatase action on autophosphorylated P-RII in *Drosophila* head extract (Aszódi et al., 1991).

We have shown that the rate of the C-subunit-mediated phosphorylation of a substrate in the intact cell was very close to that calculated from experiments with isolated enzymes using a buffer of near physiological ionic strength (Døskeland et al., 1992). Using that same buffer we found the rate of autodephosphorylation of P-RII(cAMP)₂ to be about 8-fold slower (results not shown) than in the standard low-salt buffer (Figure 2), suggesting that under physiologically relevant conditions the maximal rate of autodephosphorylation may be somewhat

slower than the maximal rate of phosphoprotein phosphatase dephosphorylation of P-RII(cAMP)₂. It should be stressed that, in the absence of a suitable quantitative experimental approach to study the rate of dephosphorylation in intact cells, estimates of such rates must be considered hypothetical. A particular experimental problem applies to P-RII(cAMP)₂, because enhanced dephosphorylation will lead to enhanced re-association with the C subunit, formation of holoenzyme, and re-autophosphorylation by an intramolecular mechanism (Rangel-Aldao and Rosen, 1976; Figure 5). Therefore the overall phosphorylation of RII may not decrease even if dephosphorylation is activated.

Buxbaum and Dudai (1989) have suggested that, owing to autophosphorylation, cA-PKII can remain partially activated for more than 10 min after cessation of a cAMP signal, implying long-term activation of cA-PKII as relevant for learning and memory; see also Schwartz and Greenberg (1987) for a review of this topic. The present data suggest that, if the kinase is actively phosphorylating substrates giving rise to C*(MgADP), the bulk of autophosphorylated P-RII may be dephosphorylated in 1 min even in the absence of phosphoprotein phosphatase activity.

Theoretical considerations

The equilibrium constants (in the forward direction) for the phosphorylation of casein by cA-PK (Shizuta et al., 1975) and of myosin light chain by myosin light-chain kinase (Geuss et al., 1985) have been reported to be 24 and 60–70 respectively. This means that it requires one to two orders of magnitude more ADP than of ATP to achieve equal rates of dephosphorylation and phosphorylation. Since cell ADP is one to two orders of magnitude lower than ATP, one will expect the forward reaction to dominate completely in the intact cell. This consideration has led investigators to ignore the reversal of the kinase reaction under physiological conditions.

The novel observation of the present study is that dephosphorylation of autophosphorylated P-RII can be linked to phosphorylation of a peptide substrate in such a way that the relative concentrations of bulk free cellular MgADP and MgATP become less important (Table 2). In fact, even when ATP was in extreme excess over ADP, the peptide substrate kemptide enhanced the dephosphorylation of P-RII(cAMP)₂ (Figure 1). Kemptide also efficiently blocked the phosphorylation of RII (Figure 5), thus shifting the equilibrium between RII and P-RII in favour of dephosphorylation. The blocking of phosphorylation of RII(cAMP)₂ by kemptide is easily explained by the latter out-competing the former for complex formation with C(MgATP) (illustrated in Table 2), since kemptide was present at concentrations well above its apparent K_m value of about 5 μ M (Whitehouse et al., 1983). The enhanced dephosphorylation in the presence of kemptide is explained (1) by the production of C*(MgADP) from C(MgATP) during kemptide phosphorylation and (2) by the fact that P-RII competes successfully with P-kemptide for C*(MgADP). This is possible because P-kemptide binds to C with about 1000 times lower affinity than kemptide, the apparent K_d of P-kemptide binding to C being about 2–4 mM (Whitehouse et al., 1983; Quamar et al., 1992). In contrast, the C subunit binds P-RII(cAMP)₂ with only moderately lower affinity than it binds RII(cAMP)₂ (Granot et al., 1980; Døskeland et al., 1993). This means that at the concentrations of kemptide

used in the present study (and presumably at the concentrations of most exogenous substrates encountered in the intact cell) phosphorylated substrates do not compete efficiently with P-RII(cAMP)₂ for binding to C*(MgADP).

The mechanism described in the present study may be important for other kinases releasing phosphorylated product before MgADP. One possible candidate reaction is auto-dephosphorylation of the Ca²⁺/calmodulin-dependent multi-functional protein kinase II. A sequential release of phosphoprotein and MgADP has been suggested for this enzyme (Kwiatkowski et al., 1990), but is not generally accepted (Hanson and Schulman, 1992). It is of interest that the presence of exogenous substrate was shown to decrease the rate as well as the steady-state level of autophosphorylation of this enzyme (Katoh and Fujisawa, 1991b).

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