Formation of Inactive cAMP-saturated Holoenzyme of cAMP-dependent Protein Kinase under Physiological Conditions*

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The complex of the subunits (RI, Ca) of cAMP-dependent protein kinase I (cA-PKI) was much more stable ($K_d = 0.25 \mu M$) in the presence of excess cAMP than previously thought. The ternary complex of C subunit with cAMP-saturated RI or RI showed devoid of catalytic activity against either peptide or physiological protein substrates. The ternary complex was destabilized by protein kinase substrate. Extrapolation from the in vitro data suggested about one-fourth of the C subunit to be in ternary complex in maximally cAMP-stimulated cells. Cells overexpressing either RI or RI showed decreased CRE-dependent gene induction in response to maximal cAMP stimulation. This could be explained by enhanced ternary complex formation. Modulation of ternary complex formation by the level of R subunit may represent a novel way of regulating the cAMP kinase activity in maximally cAMP-stimulated cells.

The cAMP-dependent protein kinase (cA-PK) differs from other kinases in having the catalytic site and the autoinhibitory site on two different subunits. The inactive cA-PK holoenzyme, when studied at nanomolar concentrations, dissociates into catalytic (C) and regulatory (R) subunits in the presence of cAMP (1). There is sparse evidence about the behavior of cA-PK at higher, more physiologically relevant, concentrations. Apparently, it is tacitly assumed that both isozymes (cA-PKI and cA-PKII) are completely dissociated by cAMP in the intact cell. The cAMP-induced decrease of fluorescent resonance transfer between microinjected Ca-FITC and RIa-TRITC (2), and between genetically encoded fluorescent Ca and RIa (3) has reinforced this notion, although such studies are not designed to tell whether the dissociation of cA-PK is complete or not (4). Recently, C/EBP null mice were shown to have increased liver RI and RII, and attenuated cAMP-stimulated hepatic gene induction (5). Protein kinase inhibitor null mice, having 50% increased muscle RI, showed deficient cAMP-stimulated CREB phosphorylation and CRE-dependent gene expression in muscle (6). We have previously observed relatively more holoenzyme-associated kinase than expected from the tissue cAMP content during the pre-replicative cAMP surge in the regenerating liver, in which both RI and RII were up-regulated (7). These observations suggest the possibility that RI or RII subunits may have a negative effect on cA-PK dissociation even at high cAMP concentrations. We used the CRE-luciferase reporter gene to probe for dissociation of cA-PKI and cA-PKII in intact cells. Nuclear translocation of the C subunit requires cA-PK dissociation and is considered a prerequisite for phosphorylation of the CREB/CREM family of nuclear transcription factors, and hence for cAMP stimulation of CRE-governed reporter gene expression (8–10). We show that cells overexpressing either hRI or hRI showed, even when maximally cAMP challenged, had decreased cAMP responsive gene induction, suggesting that cAMP produced incomplete dissociation of either isozyme in intact cells. We will also present evidence that the affinity between RI and C subunits in the presence of saturating cAMP is 1 to 2 orders of magnitude higher than hitherto assumed (11).

The presence of a substantial amount of ternary complex of cAMP, R and C in intact cells actualizes the unresolved issue of whether the cA-PK holoenzyme has any catalytic activity in the presence of cAMP (12). Several arguments have been provided in favor of this possibility. The cGMP-dependent protein kinase, which is highly homologous to cA-PK (13), is activated by cGMP without dissociation (12). The RII subunit of cA-PK type II mutagenized in the substrate motif was able to form holoenzyme without blocking the C activity (14). The cAMP-saturated cA-PKII holoenzyme was reported to be fully active (15), and this observation was linked to the fact that most cA-PK anchoring proteins (AKAPs) preferentially bind RII (16, 17). In several cases the disruption of R binding to AKAPs blocks the cAMP control of specific substrate proteins (18). This is more easily explained if cA-PK is catalytically active while physically retained in a supramolecular complex with its substrate, than if it dissociates and then translocates to the site of action in intact cells. We will also show that neither cA-PKI nor cA-PKII had significant catalytic activity against synthetic or physiological substrate under near physiological in vitro conditions.

EXPERIMENTAL PROCEDURES

Fluorescein 5-isothiocyanate (FITC) and tetramethylrhodamine-5-isothiocyanate (TRITC) were from Molecular Probes, Eugene, OR. EZ-Link Sulfo-NHS-LC-LC-Biotin was from Pierce. Streptavidin-coated 96-well “Flasplates” were from PerkinElmer Life Science. Cyclic AMP analogs were purchased from Biolog Life Science, Bremen, Germany. The heptapeptide LRRASLG (Kemptide), 99% purity, and most other chemicals were from Sigma. [5-H]Adenosine 3′,5′-cyclic phosphate and [γ-32P]ATP were from Amersham Bioscience, Buckinghamshire, UK.
Recombinant human phenylalanine 4-monooxygenase (PAH) and tyrosine 4-monooxygenase (TH) was from Dr. Torgeir Flattmark, University of Bergen, Norway. pUC7RiRs containing bovine RIIc cDNA was kindly provided by Dr. Susan Taylor, University of California, San Diego (19). pGEX-KGhRIIc and pGEX-KGhRIIs containing full-length cDNA of human R subunits were used as a substrate for GST-RIIc kindly provided by Dr. Kjetil Taskén, University of Oslo, Norway. His6hRIIs and His6hRIIs were constructed by subcloning the RIIa/RIIs cDNA into pBAD (CLONTECH). For cell transfection, RIIa and RIIb cDNAs were subcloned into pcDNA (Invitrogen). The pCMV5-Cα plasmid was a kind gift from Dr. Stanley McKnight, University of Washington, Seattle, WA. The luciferase reporter plasmid, pT81-4CRE-Luc was a kind gift from Dr. Jesper Bakke, University of Bergen, Norway. The green fluorescent protein plasmid pGFP-C1 was from CLONTECH. Buffer A is a near physiological buffer with respect to pH, ionic strength, potassium, phosphate, and magnesium concentration, and consists of 15 mM Hepes, pH 7.2, 1 mM Na2PO4, 130 mM KCl, 0.3 mM ATP, 2 mM Mg(CH3COO)2, 0.3 mM EDTA, 1 mM EDTA, 0.1 mM dithioerythritol.

Cell Culture and Transfection—HEK 293 cells were seeded at a density of 3×105 cells/ml and transfected 24 h later by calcium phosphate precipitation with reporter plasmid (0.16 μg of pT81–4CRE-Luc), various concentrations of pCMV5-Cα, pcDNA-hRIIa, or pcDNA-hRIIb. The total amount of plasmid was kept constant (2 μg) by compensating with pCMV5 empty vector. The cells were washed once with phosphate-buffered saline 24 h after transfection, lysed in 50 μl of 25 mM Tris, 1 mM EDTA, 10% glycerol, 1% Triton X-100, and 2 mM dithiothreitol for luciferase detection. Some of the lysates had received treatment with cAMP elevating agents (30 μM forskolin, 250 μM isobutylmethylxanthine) and cAMP analogs (1 mM 8-chloro-cAMP, 0.7 mM 2-monobutyryl-cAMP, and 0.3 mM 5′-O-(6-thio)-cAMP) for 30 min before harvesting. The transfection efficiency of the HEK 293 cells was determined by replacing pT81–4CRE-Luc with 0.1 μg of pGFP-C1, and visualization of green fluorescent cells 24 h after transfection.

Purification of Proteins—The Cα subunit was purified from 12 kg of bovine heart. The 10,000 × g supernatant after homogenization in 10 mM KPO4, 1 mM EDTA, 0.1 mM dithioerythritol was passed through P1-cellulose, applied to DEAE-Sepharose (1.5 liters), washed with 80 liters of 55 mM KPO4, pH 6.8, 1 mM EDTA, 0.1 mM dithioerythritol, and Cα eluted with 0.1 mM cAMP in 50 mM KPO4, 1 mM EDTA, 0.1 mM dithioerythritol. Active fractions were diluted 3-fold in water, chromatographed on Sepharose 4B, and eluted with a linear KCN gradient and washed with 600 mM KPO4 at a concentration of 0.3 mM.

Recombinant human R subunits were harvested from transformed Escherichia coli BL21, bovine R subunit from E. coli E222. Bovine RI was purified by ammonium sulfate precipitation and DEAE-Sepharose chromatography. His-tagged R was purified on Ni2+NTA chromatography. His-tagged RI and R was purified with GST-R by GSH-Sepharose. The Cα subunit was cleaved to separate GST and R was performed with GST-R still bound to resin. For final purification the R subunits were subjected to FPLC size exclusion chromatography on a column equilibrated in buffer A without ATP.

Labeling of cAMP-dependent Protein Kinase Subunits, Determination of Fluorescence Energy Transfer (FRET), and Scintillation Proximity Assay—Commercially available C-FITC and R-TRITC had decreased specific kinase activity and decreased affinity for C subunit, respectively. We therefore labeled bovine Cα subunit with FITC and bovine RI subunit with TRITC according to Adams (2). The Cα subunit was biotinylated when in holoenzyme complex to protect the R-C interaction face from modification. The cA-PK holoenzyme (2 mg/ml) was assayed on phosphoprotein substrates by phosphorylation, aliquots were mixed with sample buffer for SDS-PAGE (22), and 32P-RII and 32P-PTH detected by autoradiography after separation by SDS-PAGE. The level of C and R subunits of cA-PK in cell extracts was determined as described previously (7), except that the total level of R subunits (RI + RII) was determined by the ammonium sulfate precipitation method (23).

Estimation of the Level of R and C Subunits of cA-PK, and of C Subunit Occupation by Substrate in Intact cAMP-stimulated Cells—A thorough study (24) showed equimolar expression of the R (RI + RII) and C subunits of cA-PK in mammalian tissue, averaging 0.3 pmol/mg tissue, wet weight. This translates to an average intracellular concentration of subunits of about 0.5 μM, assuming the extracellular space to occupy 15% of the tissue, and the subunits to distribute in 70% of the intracellular space. The presently studied HEK293 cells had 3.3 pmol of R subunit/mg of protein and 3.0 pmol of C subunit/mg of protein (see first paragraph under “Results”). Assuming that the cells contained 10% protein, this value translates to about 0.3 pmol of subunit/mg of cellular wet weight, which means that the untransfected HEK293 cells had an average level of cA-PK subunit expression.

The free C subunit encounters substrates in the cell, but the % substrate saturation is unknown. To obtain an estimate, the rate of phosphorylation of PAH in vitro in the absence of competing substrates, was compared with the rate in intact, cAMP-stimulated hepatocytes (21). The phosphorylation was about 8 times slower in the intact cell, suggesting that one-eighth of the C subunit pool was accessible for PAH phosphorylation, and that at most seven-eighths of it (85–90%) was occupied by other substrates.

RESULTS

Enforced Expression of RIIα or RIIβ Attenuates cAMP-induced CRE-mediated Gene Induction—HEK 293 cells were co-transfected with pT81–4CRE-Luc and expression vector containing either RIIα or RIIβ, to study the effect of overexpressed RI and RII subunits on gene transcription via the cAMP-responsive element (CRE). The cells were stimulated with agents elevating the endogenous cAMP as well as potent cAMP analogs to ensure full saturation of the R subunits of cA-PK. It was found that cells overexpressing RIIα had about half as strong luciferase induction by cAMP agonists as control cells (Fig. 1A). A similarly blunted luciferase induction was noted in RIIα overexpressing cells exposed to potent acetoxy-methylated cAMP analogs (25, 26) at 20-fold higher concentration than required for maximal luciferase induction in control cells (not shown).

There was no evidence that RIIα expression had interfered nonspecifically with luciferase expression, since co-transfection with 400 ng of Co expression plasmid could override the effect (Fig. 1B). The effect of expressed exogenous RI and C subunits could be mutually titrated in cells not stimulated with cAMP agonists (Fig. 1, B and C), confirming that the CRE-Luc expression system responded to Co subunit and that the Co effect could be blocked by R subunit.

The luciferase induction was next compared between cells with near balanced coexpression of exogenous R and Cα and cells with moderate overexpression of Co alone. Again, cells with enforced expression of RIIα or RIIβ had lower response to cAMP challenge (Fig. 1D). This suggested that overexpressed R could inhibit the ability of exogenous (Fig. 1D) as well as of endogenous (Fig. 1A) Co to induce CRE-governed luciferase.

The observed effects of enforced expression of R subunits in cAMP-stimulated cells were statistically significant. The Wilcoxon signed-rank test showed significantly less cAMP-stimulated luciferase expression in cells overexpressing RI (p < 0.01; n = 10) or RII (p < 0.025; n = 6). When including results from cells coexpressing Co subunit, the p values were <0.001 (n = 18) for RI and <0.005 (n = 12) for RII. It is concluded that...
Cyclic AMP Saturated cA-kinase Holoenzyme Has No Kinase Activity

Since the ternary cAMP—cA-PKI complex (cAMP4,R I

RII) subunit interacts with cAMP-saturated RI that expressed less than 1% of its potential phosphotransferase activity, and possibly had no activity at all. Half-maximal inhibition of the phosphorylation was observed at 0.32 μM cAMP-saturated RI (Fig. 4B), in line with the physicochemical data indicating a submicromolar $K_d$ for the complex between Co and cAMP-saturated RI (see above). Qualitatively similar results were
tected partially against 4 ng of Co plasmid and not at all against 400 ng of Co plasmid (Fig. 1B). It also suggested that RI might be more efficient than RII in preventing the C subunit from stimulating gene transcription in cells with strongly increased cAMP levels.

CA-PKI Holoenzyme Can Form at Submicromolar Concentrations of RI and C Subunits in the Presence of cAMP—The results of Fig. 1 suggested that the RI subunit of CA-PKI could sequester the C subunit even in maximally cAMP-stimulated intact cells. This was unexpected in view of previous estimates of the dissociation constant of the complex between cAMP-saturated RI and the C subunit (11, 27). It was therefore decided to study in detail the strength of the interaction between cAMP-saturated RI and C under physiologically relevant conditions using isolated protein kinase subunits.

One approach used the ability of C-FITC to enhance the emission of RI-TRITC and RII-TRITC to quench the emission of C-FITC when at close distance (FRET) (2). The fraction of C-FITC in complex with RI-TRITC was calculated from the relative emissions at 510 and 580 nm, as detailed in Table I. Using this method, an apparent equilibrium $K_d$ of 0.24 μM was determined for the complex between RI-TRITC and C-FITC in the presence of a saturating concentration of cAMP (Fig. 2).

This $K_d$ value was surprisingly low in view of previous estimates, and could be due to enhancement of the R-C binding by the introduction of the fluorescent groups. In a second approach we used biotin-labeled Co and unlabeled RI subunit. The biotin labeling of the C subunit was performed when the C was in CA-PKI holoenzyme complex and the coupling chemistry was different from that used for the C-FITC labeling. Biotin-Co was immobilized to streptavidin-coated wells of a microplate with intrinsic solid scintillant (and scintillation proximity assay). The added RI-[3H]cAMP complex was detected only when close to the wall (bound to biotinylated C). The immobilized C subunit was estimated to be half-maximally saturated by 0.16 μM RI-[3H]cAMP (Fig. 3), confirming the high affinity between RI-cAMP and C subunit.

A third method relied on unlabeled native R and C subunits. A trace amount of Co (50 pmol) was injected into size exclusion FPLC columns equilibrated with 100 μM cAMP and various concentrations of hRI. The position where the C subunit kinase activity eluted was determined. Half-maximal shift of Co elution position occurred when the column was equilibrated with 0.25 μM hRI (data not shown). It is concluded that the Co subunit interacts with cAMP-saturated RIα with a $K_d$ in the submicromolar range.

Cyclic AMP Saturated Co- and II Lack Demonstrable Protein Kinase Activity—Since the ternary cAMP-cA-kinase complex (cAMP4,Cα, and Coα) apparently could form in intact cells (Fig. 1) and at submicromolar concentrations of c-PAK subunits (Figs. 2 and 3), the question of the catalytic activity of the ternary complex has biological importance. We investigated this possibility using several substrates. In the first series of experiments the Co-catalyzed phosphorylation of the physiological substrate phenylalanine-4-monooxygenase was studied at various concentrations of RIα in the presence of a very high concentration (100 μM) of cAMP. At the highest concentration (30 μM) of cAMP-saturated RIα studied the phosphorylation rate was inhibited more than 99% (Fig. 4A). This shows that Co in the ternary complex with CAMP-saturated RI expressed less than 1% of its potential phosphotransferase activity, and possibly had no activity at all. Half-maximal inhibition of the phosphorylation was observed at 0.32 μM CAMP-saturated RIα (Fig. 4B), in line with the physicochemical data indicating a submicromolar $K_d$ for the complex between Co and cAMP-saturated RI (see above).
The cAMP-saturated cA-kinase Holoenzyme Has No Kinase Activity

### TABLE I
Determination of the fractional association of RIα-TRITC (RIα) and Ca-FITC (Ca) by fluorescence resonance energy transfer analysis

| Incubate content | cps 510 nm | cps 580 nm | cps 580 nm (RIα only) | Ratio 510 nm/580 nm (Ca+RIα) | Fractional association (r_{f

| 30 nm Ca | 14,500 | 2,260 | 0 | | |
| 50 nm RIα | 153 | 2,820 | 2,820 | 5.14 r_{f0} | 0.00 |
| Σ 50 nm RIα + Ca | 14,653 | 5,080 | 2,820 | 5.05 r_{f0} | 0.07 |
| 50 nm RIα + Ca + cAMP | 14,000 | 4,952 | 2,770 | 3.70 r_{f0} | 1.0 |
| 50 nm RIα + Ca | 11,900 | 5,075 | 3,220 | |
| 500 nm RIα | 128 | 22,400 | 22,400 | 0.647 r_{f0} | 0.00 |
| Σ 500 nm RIα + Ca | 14,628 | 24,660 | 22,400 | 0.496 r_{f0} | 0.86 |
| 500 nm RIα + Ca + cAMP | 11,800 | 25,638 | 23,800 | 0.471 r_{f0} | 1.0 |

**Fig. 2.** The association of FITC-labeled Ca subunit with cAMP saturated TRITC-labeled RIα subunit of cAMP kinase determined by fluorescence resonance energy transfer. The data shown are from a typical experiment conducted like that shown in Table I, with constant concentration (30 nM) of Ca-FITC and increasing concentrations of RIα-TRITC at 50 µM cAMP. The fractional association was determined as described in Table I, and was half-maximal at 0.24 µM of added RIα-TRITC.

obtained when the Ca activity was determined with tyrosine-4-monoxygenase as the substrate, using autoradiography to detect the phosphorylated protein (Fig. 5). The phosphorylation of tyrosine-4-monoxygenase in the presence of cAMP (100 µM) was inhibited by at least 95% also by RIα (Fig. 5).

RIα and RIβs (10 µM) inhibited the kinase to a similar degree whether the free cAMP concentration was 50, 100, 300, or 600 µM. This suggested that 50–100 µM cAMP, as routinely used, could saturate cA-PKI and II holoenzyme nearly completely.

Although cAMP-saturated cA-PKI or II holoenzyme had insignificant activity toward large physiological protein substrates (Figs. 4 and 5), the possibility remained that they could phosphorylate smaller substrates (15). In our hands, RIα (Fig. 6), as well as RIβs (Fig. 7), could inhibit Kemptide phosphorylation nearly completely in the presence of cAMP. The inhibition was reversible and similar whether bovine or human RIα was used, and whether the R subunit was a GST fusion protein, a thrombin-cleaved product of the latter, or was polyhistidine-tagged (not shown).

Estimation of the Stability of the Ternary Complexes of cAMP-RIα-Ca and cAMP-RIβs-Ca at Assumed Physiological Substrate Concentration—A final experiment was designed to test if more RIα was required to inhibit the kinase activity when the substrate concentration was increased, as expected if substrate and R subunit compete for binding to the C subunit. Elevation of the Kemptide concentration from 8 µM through 70 µM to 140 µM increased the concentration of cAMP-saturated RIα required for half-maximal inhibition of Ca from 0.47 µM through 0.81 µM to 1.2 µM (Fig. 6). At 100 µM Kemptide about 1 µM RIα was required to half-maximally inhibit the kinase (Fig. 7). The K_m value for Kemptide was determined to be 11 µM, and at 100 µM Kemptide the activity was about 90% of V_max, suggesting 90% occupation of the C subunit by substrate (not shown). It is concluded that 1 µM cAMP-saturated RIα or RIβ is sufficient to achieve 50% kinase inhibition, even at substrate concentrations 10 times above the K_m value. The data were obtained with RIα and RIβ in the dephospho-form. In separate experiments using RIα phosphorylated by the C subunit, a higher concentration of cAMP-saturated RIα was required to inhibit the C subunit (not shown). From previously published data it can be estimated that the C subunit in cAMP-stimulated hepatocytes is at most 90% saturated with substrate, and that the concentrations of C subunit and R subunit (RI + RI) is about 0.5 µM in the average cell (see the last paragraph under “Experimental Procedures”).

Determination of the fractional association of RIα-TRITC (RIα) and Ca-FITC (Ca) by fluorescence resonance energy transfer analysis

The FITC-labeled Ca subunit (30 nM) and the TRITC-labeled bRIα subunit (50 or 500 nM) were excited at 490 nm, and the emission monitored (cps) at 510 and 580 nm. The emission at 510 nm was due to Ca alone, since RIα did not emit at this wavelength. At 580-nm Ca had significant emission (15.6%) of that at 510 nm which was subtracted to obtain the emission due to RIα only (column 3 from the right). The ratio of emission 510/580 nm (CaRIα) is shown for the separated subunits (r_{f0}), and for combined subunits in the presence (r_x) and absence (r_{rf0}) of 50 µM cAMP. The r_{f0} represents the completely dissociated state (fractional association = 0), and r_{rf0} the completely associated holoenzyme state (fractional association = 1.0). The fractional association observed in the presence of cAMP was determined as shown in the right hand column. The experiment was conducted at 25 °C in buffer A.
Inhibition of Ca catalyzed phosphorylation of PAH by RIα at saturating concentration of cAMP. Panel A shows the time kinetics of PAH (4 μM) phosphorylation by 0.5 μM Ca subunit in the absence of RIα (○), and in the presence of 1.25 μM (□) or 30 μM (△) RIα. Panel B shows the inhibition of C subunit activity by increasing concentrations of cAMP-saturated RIα. Incubation conditions were as described in the legend for Table I, except that the temperature was 37 °C, and [32P]ATP (2.5 μCi/ml) was present. The data were based on initial kinase activities, under the condition of the experiment in panel A (○, □, and △) or experiments conducted with 0.3 μM Ca subunit (●), and incubation times from 1 to 12 h. Half-maximal inhibition of PAH phosphorylation was observed at 0.32 μM RIα. At 30 μM RIα the PAH phosphorylation rate was 0.8% of that observed in the absence of RIα.

The ternary complex was more stable than previously recognized, the question of its catalytic activity becomes biologically important. An intriguing possibility is that R-C holoenzyme associated with scaffolding protein (AKAP), and thereby in immediate contact with AKAP-tethered substrate (16, 17), may be retained in an active holoenzyme complex close to the substrate upon cAMP activation (15). The present study failed to demonstrate any significant kinase activity of Ca in ternary complex with cAMP and RIα toward the heptapeptide Kemptide or physiological substrates (phenylalanine-4-monoxygenase and tyrosine-4-monoxygenase). Neither cAMP-saturated Ca-PKII holoenzyme show demonstrable catalytic activity. These results were reproduced with a number of different preparations of R and C subunits, and under a variety of conditions, including close to physiological with respect to temperature, pH, and ionic strength. We conclude that dissociation is a prerequisite for both Ca-PKII and Ca-PKI to catalyze substrate phosphorylation, at least under physiologically relevant conditions in vitro. Obviously, cAMP-saturated Ca-PKI and Ca-PKII differ from cyclic nucleotide-saturated CaMP-dependent protein kinase in this respect (12). An early report (11) suggested that the ternary complex between cAMP, RI, and C subunit had about 15% of the activity of the free C subunit. A more recent study (15) found the ternary complex between cAMP, RI, and fluorescein-labeled C subunit to have full catalytic activity toward Kemptide. A possible explanation of this discrepancy may be that C subunit labeled with fluorescein-succinimidyl ester (15) has poor ability to interact with RI subunit (4) and may have subtly altered interaction with RI as
well, allowing cA-PKII holoenzyme formation without kinase inhibition. It is known that point mutations of Ca can affect binding to either RI or RII, without interfering with the catalytic activity (28).

The ternary complex of C subunit with cAMP and R was destabilized by protein kinase substrate (Fig. 6). We envisage therefore that substrate depletion due to phosphorylation, by allowing more C subunit to be sequestered in ternary complex, may act as a negative feedback mechanism of kinase activity. Experimental verification of this possibility is hampered by the instability of the FRET signal used to monitor cA-PK dissociation in intact cells, due to photobleaching and nuclear translocation of the C subunit. We note, however, that Zaccolo et al. (3) reported the FRET signal typical of cA-PKII dissociation to decrease with time in some Chinese hamster ovary cells continuously exposed to a maximal cAMP stimulus.

Extrapolation of the in vitro data suggests that about one-fourth of the C subunit can be sequestered as inactive, cAMP-saturated cA-PK (ternary complex) in the average cAMP-stimulated cell. This allows a novel avenue for control of the cA-PK activity in maximally cAMP-stimulated cells, through regulation of the R subunit level. Up-regulation of R subunit will sequester more C subunit as inactive complex, and down-regulation of R will release kinase from the ternary complex. Such regulation will not be possible if it is assumed that down-regulation of R will release kinase from the ternary complex in the cytoplasm. In retrospect, ternary complex formation may explain the attenuated cAMP-stimulated gene induction in C/EBP (7). Similarly, ternary complex formation may explain the at-

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