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Lipolytic Membrane Release of Two Phosphatidylinositol-Anchored cAMP Receptor Proteins in Yeast Alters Their Ligand-Binding Parameters

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Two new cAMP-binding proteins have been discovered recently in *Saccharomyces cerevisiae*. They are genetically distinct from the regulatory subunit of cytoplasmic cAMP-dependent protein kinase A and are distinguished from the latter, in addition, by their anchorage through phosphatidylinositol-containing lipid and glycolipid structures to mitochondrial and plasma membranes, respectively (Müller and Bandlow, 1989 *Biochemistry* 28, 9957–9967, 1991, *Biochemistry* 30, 10181–10190). A nutritional upshift induces the cleavage of the anchor by a phospholipase C (Müller and Bandlow, 1993, *J. Cell Biol.* 122, 225–236). To test the idea that anchorage by (glycosyl)phosphatidyl-inositol influences cAMP-binding and has a regulatory function, we analyzed ligand binding to the two purified cAMP receptors (46,000 and 54,000 Da) in comparison to the regulatory subunit of the cytoplasmic protein kinase A (52,000 Da). We find that lipolytic cleavage of the two membrane anchors by phosphatidylinositol-specific phospholipases C and D results in significantly higher association and lower dissociation rates of cAMP, thus leading to a dramatic increase in ligand affinity of the two cAMP receptors. Use of cAMP analogues identifies two different cAMP-binding centers in each membrane-embedded protein, one of which is noticeably affected by the cleavage of the anchor. In both phosphatidylinositol-anchored cAMP receptor proteins a single Trp residue in one of the binding centers is photoaffinity-labeled by 8-N_3-cAMP, whereas two amino acids, Trp and Tyr, are modified after lipolytic removal of the anchor. The differences in the labeling patterns are interpreted as to result from a conformational rearrangement induced by the cleavage of the anchor. Together with the increased affinity to the ligand these changes document alterations of the properties and folding structure of lipid-anchored proteins following cleavage of the PI-containing anchor by specific phospholipases and provide the first molecular evidence for a regulatory role of the anchorage by a lipid structure. The cytoplasmic regulatory subunit of yeast protein kinase A is not photolabeled to a significant extent under any condition.

In all eukaryotic cells and tissues, cAMP-dependent effects have been found to be mediated by PKA[2] (1). Two isoforms of tetrameric PKA holoenzymes have been detected, called type I and type II (2, 3). They are distinguished by their regulatory subunits, accordingly named RI and RII, which differ from one another in a number of parameters. Among others they can be discriminated with respect to their primary structure, the affinities for the respective C subunits (4), and their autophosphorylation potential (4–6) and in the on and off rates of cAMP and cAMP analogue binding (7–10). Both, type I and type II R subunits, bind 2 mol of cAMP per mol of subunit with about equal affinity to either center. cAMP analogues, on the other hand, typically exhibit binding preferences to the one center (or to one type of subunit) as compared to the other and can be used to classify an R subunit and to characterize its two binding centers (11), the more N-terminal being called site A and the other

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[2] Abbreviations used: (G)PI, (glycosyl)-phosphatidylinositol; (G)PI-PLC (D), (glycosyl)-phosphatidylinositol-specific phospholipase C (D); PC, phosphatidylcholine; PKA, cAMP-dependent protein kinase A (soluble, cytoplasmic form); R (C), regulatory (catalytic) subunit; TPA, 12-myristoyl-13-acetylphorbol; HPLC, high-performance liquid chromatography; TLE, thin-layer electrophoresis; Mops, 4-morpholinepropanesulfonic acid; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; BSA, bovine serum albumin; PBS, phosphate-buffered saline; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; Hepes, 4-(2-hydroxyethyl)1-piperazineethanesulfonic acid.

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one site B. Only the saturation of all four centers of the inactive holoenzyme complex by the ligand leads to the dissociation of two (active) C subunits (4).

Yeast has been shown also to harbor a cAMP-dependent protein kinase of the R_2C_2 type (12). It is located in the cytoplasm and has been classified as belonging to type II (3). Its R subunit is encoded by a single nuclear gene (*BCY1*) (13). Three different genes for C subunits (*TPK1, TPK2, TPK3*) have also been identified in yeast (14).

In the course of the analysis of cAMP-dependent effects, which are not mediated by cytoplasmic PKA, we have recently identified two additional cAMP receptor proteins in yeast: one at the outer face of the inner mitochondrial membrane (15–19) and the other one at the periplasmic face of plasma membranes (20, 21). Both of them are encoded by (a) gene(s) different from *BCY1* because they can be photoaffinity labeled in *bcy1*-disruption mutants. They can be discriminated further from the R subunit of cytoplasmic PKA, apart from topology, also by the lack of immunological cross-reactivity and by different peptide degradation patterns (21). Particularly striking and discriminatory is the finding that both behave as integral membrane proteins. They are anchored to their resident membranes by PI-containing lipidic anchors which differ from one another, however, in the nature and arrangement of their constituents. The cAMP receptor from plasma membranes has a typical GPI anchor (22), whereas the one from mitochondria contains PI and ethanolamine but lacks carbohydrate (17, 18). Neither of the two cAMP-binding proteins activates a catalytic protein kinase subunit upon the mere binding of cAMP. However, in the case of the mitochondrial isoprotein, a correlation has been found between the release of the cAMP-binding protein from the inner membrane in soluble form by an endogenous PL and the emergence of cAMP-dependent protein kinase activity in the mitochondrial intermembrane space. This kinase phosphorylates both intrinsic mitochondrial proteins and exogenous acidic substrates (16, 19). For the cAMP-binding ectoprotein from plasma membranes we have recently shown that lipolytic cleavage of the GPI membrane anchor is a natural process which is induced in response to a shift of cells from nonfermentative growth to glucose medium (22a). To provide complementary evidence in support of a regulatory role for anchorage to the membrane of the two new cAMP receptor proteins by a lipid structure, we compare the ligand-binding to the anchor-containing and the anchor-free forms. We report here that lipolytic anchor cleavage changes the properties of the two cAMP-binding proteins in that it leads to an increased affinity of the hydrophilic versions to the ligand.

MATERIALS AND METHODS

Materials. With the exception of 8-N_3-[^{32}P]cAMP, which was bought from ICN (Eschwege), all radiochemicals and scintillation cocktail ACS II were purchased from Amersham-Buchler (Braunschweig); cAMP, cAMP analogues, TPA, lipids, and dipeptides were provided by Sigma (Deisenhofen) (the cyclic nucleotides were stated by the manufacturer to be 97–99% pure); detergents, IBMX, cyclic nucleotide-specific phosphodiesterase (bovine heart), and purified PI-PLC (*Bacillus cereus*) were bought from Boehringer (Mannheim); purified GPI-PLC (*Typanosama brucei*) was the kind gift of P. Overath, (Tübingen); crude GPI-PLD (rabbit serum) and purified PI-PLC (*Bacillus thuringiensis* and *Staphylococcus aureus*) were kindly donated by W. Gutensohn (Munich).

Purification of cAMP-binding proteins. The cytoplasmic fraction was prepared from the postmitochondrial supernatant by centrifugation (200,000*g*, 60 min, 4°C) and the resulting supernatant (5 mg protein per ml) dialyzed extensively against binding buffer (20). Mitochondria and plasma membranes were solubilized in 0.5% deoxy-cholate and 0.75% octyl glucoside, respectively (15 min on ice followed by centrifugation at 150,000*g*, 30 min). The cAMP-binding proteins were partially purified by affinity chromatography. To remove cyclic nucleotides, the eluate was incubated with phosphodiesterase (2.5 U/ml, 30 min, 22°C) and, after addition of 0.1 mM IBMX, centrifuged through a Sephadex G-25 column equilibrated with Mops buffer (see below). The PEG 4000 (8% final concentration, 30 min, 4°C) precipitate (10,000*g*, 15 min, 4°C) was washed successively with 1% and 0.2% PEG 4000 and dissolved at 1 mg/ml in 25 mM Mops/KOH (pH 7.4), 0.5 mM EDTA, 1 mM DTT, 20 mM KCl, 100 μM PMSF, soybean trypsin inhibitor (10 μg/ml), 10 μM leupeptin, 1 mM iodoacetamide, 0.2 mg/ml BSA, 5% glycerol (buffer A) containing the appropriate detergent (0.2–0.3%). The anchor-containing cAMP-binding proteins were further enriched by phenyl-Sepharose chromatography. To 25 μg protein 1350 μl of PBS (containing 175 mM NaCl, the final concentration of detergent never exceeded 0.005%) and 100 μl of phenyl-Sepharose beads (washed prior to use in PBS containing 0.001% Nonidet-P40) were added. After 15 min at 25°C the beads were collected (15,000*g*, 1 min), washed successively with 1 ml of PBS containing 0, 0.005, 0.05, and 0.2% (for the mitochondrial cAMP-binding protein) or 0.75% (for the plasma membrane cAMP-binding protein) of the respective detergent. The final supernatant was precipitated by PEG, washed and dissolved as described above. All samples were frozen immediately in liquid N_2 and stored at −70°C.

Reconstitution into liposomes. Detergent-solubilized mitochondrial or plasma membrane cAMP-binding proteins (1.5 μg) were sonicated (five times, 10 s, 4°C, ultrasonic bath) in the presence of 0.25 mg each of phosphatidylserine, phosphatidylcholine, and phosphatidylethanolamine in 100 μl of binding buffer and incubated (15 min, 4°C). After dilution with 10 ml of binding buffer and further incubation (30 min, 4°C) the mixtures were centrifuged (250,000*g*, 30 min, 4°C). The liposomes were washed two times with binding buffer, suspended in 100 μl of buffer A, and immediately used for binding tests.

Kinetics and competition of [^{3}H]cAMP binding. Purified detergent-solubilized or reconstituted cAMP-binding protein (0.25 μg) was incubated with 15 pmol of [^{3}H]cAMP (100 nCi) in 100 μl of binding buffer in the absence or presence of 0.2% deoxycholate (mitochondrial protein) or 0.75% octyl glucoside (plasma membrane and cytosolic protein) at 4°C. For studying the association kinetics the binding reaction was terminated by precipitation (see below) after various periods of time following the addition of [^{3}H]cAMP. Dissociation kinetics was studied by supplementation of the mixture with 150 nmol of unlabeled cAMP after 30 min preincubation with [^{3}H]cAMP (4°C) and precipitation at the times indicated. For displacement studies under equilibrium conditions, the incubation with [^{3}H]cAMP (14 h) was performed in the presence of increasing concentrations of cAMP or cAMP analogues covering a 2- to 10,000-fold concentration range. The incubations were terminated by precipitating the proteins with 400 μl of ice-cold 10% PEG 4000 (30 min, 4°C). Two 200-μl aliquots from each sample were diluted with 1 ml of ice-cold 8% PEG 4000 and filtered through Millipore membranes (HAWP, 0.45 μM) under constant vacuum, washed with 5 ml of cold 8% PEG 4000 in binding buffer and dried filters counted in 5 ml of scintillation fluid. Specific binding of [^{3}H]cAMP was calculated as the difference between total and unspecific binding (measured in the presence

of 0.1 mM unlabeled cAMP). Each point gives the mean of three preparations of cAMP-binding protein with four assays each.

Proteolytic digestion of photolabeled cAMP-binding protein. After separation of 8-N_3-[^{3}H]cAMP-labeled proteins by SDS–PAGE, the proteins were extracted from the minced gel pieces (two times with five gel volumes of 0.1 M Hepes/KOH (pH 8.0), 0.1% SDS, 6 M urea and mixing overnight at 56°C) and the gel fragments removed by centrifugation. Combined dialyzed supernatants were concentrated (Speedvac) and incubated in 1 ml pronase (25 mg/ml), 0.1 M Hepes/KOH (pH 8.0), 15 mM $CaCl_2$, 0.1% Triton X-100 (10 h, 50°C). Following the addition of a second aliquot of pronase (to 40 mg/ml total) and SDS (to 0.5%), digestion was continued for 7 h at 50°C. Subsequently proteins were precipitated by PEG 4000 (8% final concentration), the supernatant was filtered through an ultrafiltration membrane (exclusion limit 1000 Da, Diaflo, UM-2), and the filtrate concentrated (Speedvac).

TLE analysis of photoaffinity-labeled amino acids. Dried pronase digests were dissolved in 25 μl electrophoresis buffer (buffer A: 88% formic acid/glacial acetic acid/water, 50/56/1794 [v/v], pH 1.9; buffer B: pyridine/glacial acetic acid/water, 10/1/89.5, pH 5.5, containing 20% [v/v] acetone) and applied to a 0.25-mm silica gel G plate (2.5 μg digested protein) and electrophoresed (90 min, 500 V). Regions (0.5 cm wide), scraped off the plate, were counted for radioactivity in a liquid scintillation counter. Photoaffinity-labeled standard amino acids (5 pmol) (see below) were run in parallel on the same plate. The R_f values represent the means of three independent experiments obtained with different membrane preparations of each cAMP-binding protein.

HPLC analysis of photoaffinity-labeled amino acids. Dried pronase digests (1500–3000 dpm) were suspended in 50 μl of ethyl acetate/methanol (1/1) and analyzed on a Waters Associates Model 204 liquid chromatograph equipped with a DuPont Zorbax ODS (25 cm × 4 mm) column with a 55°C water jacket. The column was eluted isocratically at 2 ml/min by using 32% acetonitrile and 68% 0.02 M sodium acetate (pH 4.5) containing 1% acetonitrile. Eluted radioactivity was monitored continuously. Values, taken at 30-s intervals, of one typical experiment, repeated three times with similar results, are shown.

Preparation of photoaffinity-labeled amino acids. Glycine or the dipeptides Gly–Tyr and Gly–Trp (10 pmol) was photoaffinity-labeled with 10 nmol of 8-N_3-[^{3}H]cAMP in 25 mM Hepes/KOH (pH 6.2), 0.5 mM EDTA, 0.5 mM 2-mercaptoethanol, 100 μM 5'-AMP, 5% glycerol in a total volume of 50 μl under irradiation with uv_{254} in the wells of a ceramical serological plate (4°C, 30 min). After addition of 10 μmol of unlabeled cAMP, the dipeptides were digested with pronase. Gly (modified at the amino group), Trp, and Tyr (modified at the aromatic side chains) were electrophoresed as described above.

Miscellaneous procedures. Published procedures were used for growth and subcellular fractionation of yeast (strain ABYS-1; (17, 20, 23), photoaffinity labeling with 8-N_3-[^{3}H]/[^{32}P]cAMP (15, 20), affinity purification on cAMP Sepharose (24), incubation of mitochondria with Ca^{2+} plus TPA (17), treatment of cAMP-binding proteins with (G)PI-PLC/D (20), TX-114 partitioning (21, 25), determination of soluble (28) and membrane protein (26), and SDS–PAGE (27).

RESULTS

Ligand Binding to Three cAMP Receptor Proteins

To study cAMP-binding to two cAMP receptors, lipid-anchored to yeast mitochondria and plasma membranes, respectively, and to the R subunit of cytoplasmic PKA for comparison, all three proteins were purified from the respective compartment by affinity and hydrophobic interaction chromatographies. Figure 1 summarizes the purification scheme of the three different cAMP receptors. In intact spheroplasts one major protein with molecular mass 54 kDa can be affinity labeled with membrane-impermeable, photoreactive 8-N_3-[^{32}P]cAMP (lane 2 of Fig. 1) (cf. 19). The labeling can be competed for by unlabeled cAMP, reflecting the specificity of the binding (lane 1). Cellular subfractions were isolated and mitochondria, plasma membranes, and cytoplasmic supernatant fractions controlled for low mutual contamination as described (14, 19, 20) and photoaffinity labeled. Several radioactive protein bands occur in these fractions, some of which may be due to unspecific labeling or degradation (lanes 3 to 5). After cAMP–Sepharose (lanes 6 to 8) and—in the case of the two anchor-containing proteins—phenyl–Sepharose affinity chromatographies, only one isoprotein is photoaffinity labeled in each subfraction: a 54-kDa protein can be purified from plasma membranes (lane 9), a 46-kDa protein from mitochondria (lane 10), and primarily a 52-kDa protein from the cytoplasm (lane 8). These compartment-specific labeling patterns reflect the absence of cross-contamination and prove that the three polypeptides represent individual entities and are not derived from the largest one by proteolysis. The cytoplasmic cAMP-binding R subunit (lanes 5 and 8) is only poorly labeled despite extensive dialysis. A signal is detectable only upon prolonged exposure of the fluorogram (see below for discussion). The partially purified materials, the radiolabeled derivatives of which are displayed in lanes 8 to 10, were routinely used in the subsequent ligand-binding studies. (The enrichments achieved were about 120-fold with the cytoplasmic protein

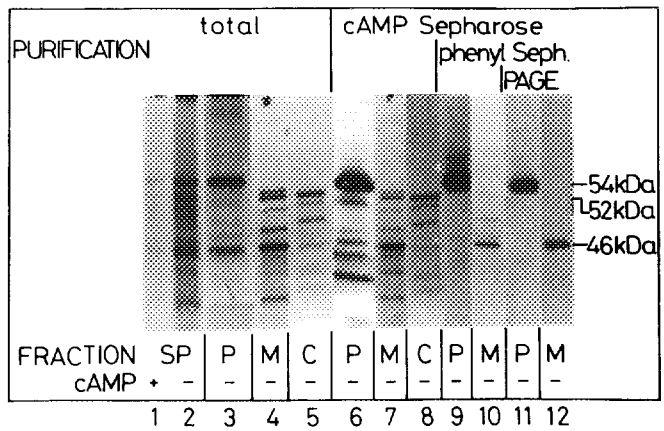

FIG. 1. Purification of cAMP-binding proteins. Seventy-five micrograms each of spheroplasts (SP), plasma membrane (P), mitochondrial (M), and cytoplasmic (C) fractions were photoaffinity labeled with 8-N_3-[^{32}P]cAMP in the absence (lanes 2–5) or presence (lane 1) of excess unlabeled cAMP and analyzed by SDS–PAGE and fluorography (total, lanes 1–5). Unlabeled cAMP-binding proteins were enriched by cAMP Sepharose affinity chromatography (lanes 6–8) followed by phenyl–Sepharose chromatography (phenyl Seph., lanes 9 and 10) and subsequently photoaffinity labeled and analyzed by SDS–PAGE and fluorography. The radiolabeled material migrating at 54 kDa in lane 9 and at 46 kDa in lane 10, respectively, was eluted from the gel and analyzed again by SDS–PAGE and fluorography (PAGE, lanes 11 and 12). The molecular masses on the right margin were derived from marker proteins run in parallel.

over the homogenate, 180-fold with the plasma membrane, and about 50-fold with the mitochondrial proteins, respectively. These protein fractions were determined by Scatchard analyses to bind 4 to 5 pmol/mg [^{3}H]cAMP in the case of the cytoplasmic, 110 pmol/mg with the plasma membrane and 65 pmol/mg with the mitochondrial cAMP receptor (not shown); the pure proteins theoretically would bind about 40 nmol/mg each.) The anchor was cleaved by treatment with PLs at this stage of purification where indicated and the hydrophilic, anchor-free form separated from the anchor-containing, uncleaved material by TX-114 partitioning. Lanes 11 and 12 display the results of further purification of the anchor-containing membrane proteins by additional SDS–PAGE and reisolation of the radiolabeled band. This material was used for the determination of the amino acid residues modified by photoaffinity labeling (see below).

cAMP Binding Kinetics

Purified material of the three proteins (corresponding to Fig. 1, lanes 8 to 10) was used to study the rate of dissociation and association of [^{3}H]cAMP. For measuring dissociation the cAMP receptor proteins were preloaded with ^{3}H-labeled ligand, and the reaction was started by the addition of a 1000-fold excess of unlabeled cAMP. Dissociation rates are displayed as the logarithmic plot of the ratio of ligand concentration bound at time (t) to that bound initially under equilibrium conditions vs time. Comparison of Figs. 2A and 2B reveals that dissociation rates of the ligand from the anchor-containing cAMP receptors from plasma membranes and mitochondria are similar. They are about the same with detergent-solubilized proteins or after reconstitution into liposomes (absence of detergent, Figs. 2A and 2B) or when membrane vesicles are analyzed (not shown). However, velocities of ligand dissociation from the anchor-containing cAMP-binding proteins are significantly faster than from the cytoplasmic R subunit (measured both in the presence and absence of detergent) (Fig. 2C).

In order to examine the possibility that anchor cleavage influences cAMP-binding parameters and may have regulatory implications we studied, whether the lipolytic removal of the anchor has any effect on k_{off} and k_{on}. Cleavage of the anchor of the cAMP receptor from plasma membranes was achieved by either extrinsic (G)PI-PLC and D (Fig. 2A) or, in the case of mitochondria, by PI-PLC or by activating an intrinsic PL with phorbol ester in the presence of ATP and Ca^{2+} (17–19) (Fig. 2B). As expected, this treatment has no significant effect on the kinetics of ligand dissociation from the cytoplasmic R subunit (Fig. 2C). By contrast, cleavage of the two anchor-containing cAMP receptor proteins with extrinsic PI-PLC (*B. cereus*) and GPI-PLD (rabbit serum) as well as—in the mitochondrial case—incubation with Ca^{2+}, ATP, and TPA leads to four- to fivefold reduction of the dissociation rates which, after cleavage with GPI-PLD or with the intrinsic PL, approach that observed with the cytoplasmic R subunit. These results demonstrate that anchor cleavage increases the affinity for the ligand.

In order to confirm these conclusions and to calculate dissociation constants, association kinetics were measured. The reaction was treated as being pseudo-first order. Figs. 2D–2F display the logarithmic plots of the ratio of ligand concentration bound under equilibrium conditions to the difference of the ligand concentrations bound under equilibrium conditions and bound at each time point (t) vs time. It can be seen again that anchor-containing forms (either detergent-solubilized or reconstituted into liposomes) exhibit two- to threefold lower velocities of ligand association as compared to the same proteins solubilized by lipolytic cleavage and to the R subunit of cytoplasmic PKA (in the presence of detergent). Both association and dissociation plots display nonlinear curves, especially in the cases of the membrane-bound receptor proteins. This kinetics points to the occurrence of either heterogenous cAMP-binding sites or to cooperativity between two (or more) similar binding sites rather than to a bimolecular reaction involving only one type of binding center.

Table I compiles the on and off rates and the dissociation constants obtained with the three cAMP receptor proteins and compares them with respect to the influence of lipolytic cleavage of the anchor. It is evident that removal of the anchor, rather than solubilization *per se* simultaneously accelerates association and diminishes dissociation velocities. This results in an increase of affinity to the ligand by about one order of magnitude, suggesting significant structural rearrangement of the cAMP receptor proteins at or around the ligand-binding centers upon lipolytic cleavage of the C-terminally attached anchors.

Nucleotide Specificity

To explore the possibility further that two different cAMP-binding sites might be present in the membrane-bound cAMP receptor proteins as suggested by the nonlinear binding kinetics, we tested nucleotide specificity of binding to the anchor-containing proteins. cAMP analogues differentiate between the two centers, A and B, in both type I and type II receptor proteins in mammals (7, 9, 11, 29). We studied the efficiencies of analogues to displace [^{3}H]cAMP from prelabeled receptors. The ranking of the competition efficiencies of the analogues is the same with the two anchor-containing proteins from plasma membranes (Fig. 3A) and mitochondria (Fig. 3B), arguing that the two receptors have very similar binding parameters. It can be seen that analogues, modified at the N^6-position of the adenine ring, are very efficient in displacing [^{3}H]cAMP. In vertebrates these analogues exhibit a preference of

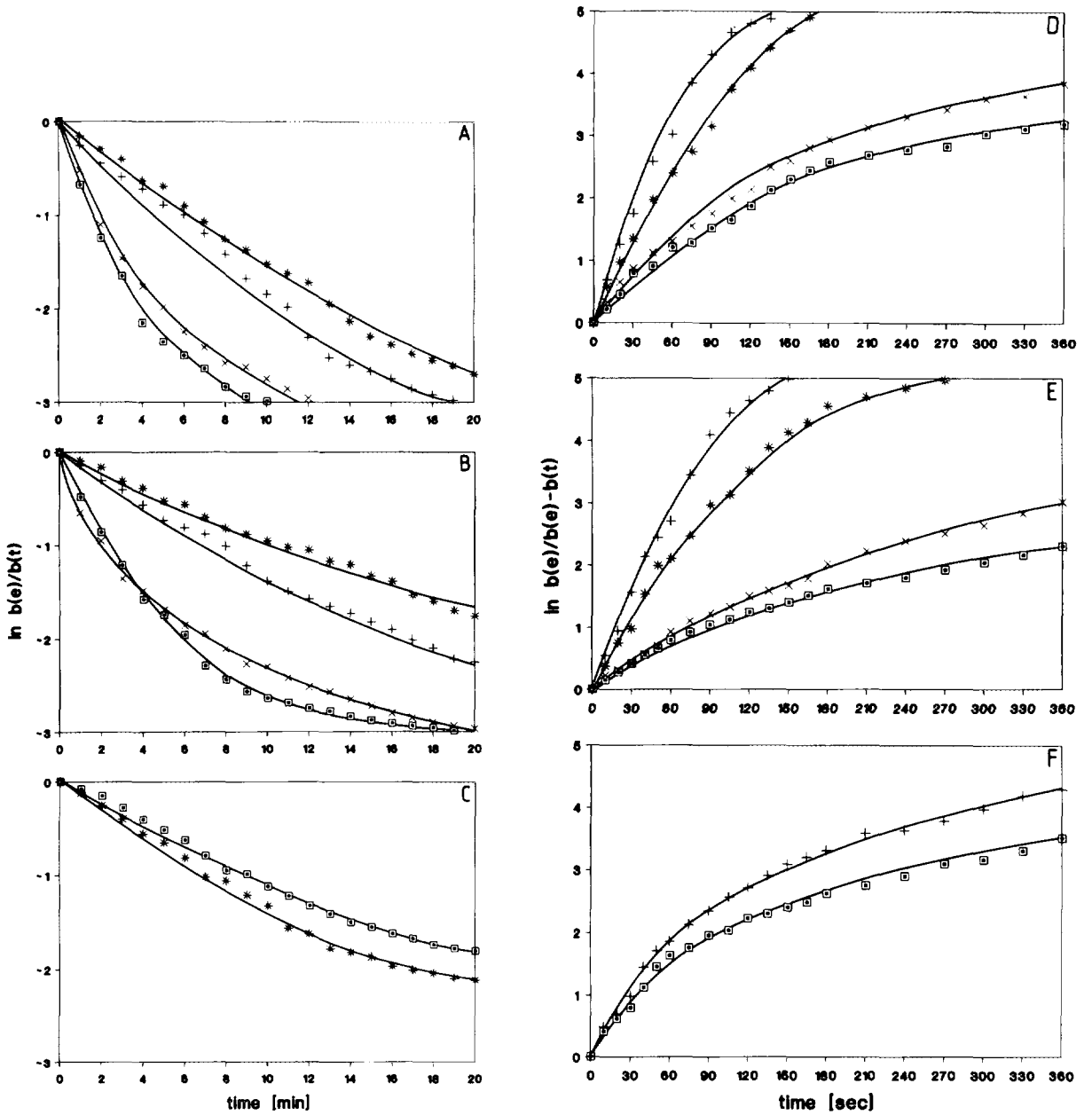

FIG. 2. Influence of anchor cleavage on the kinetics of dissociation (A–C) and association (D–F) of [^{3}H]cAMP with cAMP-binding proteins from plasma membranes (A, D), mitochondria (B, E), and cytoplasm (C, F). Anchor-containing, detergent-solubilized (×) and reconstituted (⊡) as well as anchor-free forms of the membrane proteins were analyzed. The anchor of the cAMP receptor from plasma membranes was cleaved by GPI-PLD (*) or PI-PLC from *B. cereus* (+) (A, D). The mitochondrial isoprotein was cleaved by PI-PLC from *B. cereus* (+) or by incubation with Ca^{2+} plus TPA in the presence of an ATP-regenerating system (*) (B, E). Affinity-purified cytoplasmic R subunit (C, F) was studied in the absence (⊡) or presence (✳) of 0.5% octylglucoside. Ligand dissociation (A–C) was started by addition of excess unlabeled cAMP, ligand association (D–F) was started by addition of [^{3}H]cAMP. Both reactions were terminated after the time periods indicated. $b(t)$, specific binding after time (t); b(e), specific binding at equilibrium.

binding to site A. Of C-8-modified analogues, e.g., 8-N_3-cAMP and particularly cGMP and cIMP, higher concentrations are required to replace labeled cAMP. In vertebrates these substances preferentially associate with site B of RI and RII (30).

Affinity-Labeled Amino Acids of the Lipid-Modified cAMP Receptor Proteins

To identify residues which interact with the ligand, we photolabeled the two detergent solubilized, anchor-con-

TABLE I

cAMP-Binding Parameters of Membrane-Bound and Soluble cAMP-Binding Proteins

Origin	Treatment	K_d (nM)	k_{on} (min^{-1} nM^{-1})	k_{off} (min^{-1})
Plasma membranes	Control	4.82 ± 1.32	3.54 ± 0.88	14.53 ± 3.89
	PI-PLC	0.32 ± 0.18	9.78 ± 1.31	3.16 ± 0.51
	GPI-PLD	0.45 ± 0.13	11.63 ± 2.10	5.26 ± 0.96
Mitochondria	Control	2.91 ± 0.92	3.13 ± 0.34	9.12 ± 2.19
	PI-PLC	0.22 ± 0.11	7.89 ± 1.85	1.71 ± 0.35
	Ca^{2+}/TPA	0.29 ± 0.08	7.92 ± 2.44	2.26 ± 0.59
Cytoplasm	Control	0.41 ± 0.08	3.15 ± 0.71	1.29 ± 0.48
	PI-PLC	0.52 ± 0.07	3.76 ± 0.46	1.94 ± 0.67

Note. k_{on} and k_{off} of the anchor-containing (Control) and anchor-free cAMP-binding proteins were calculated from the initial velocities of the association and dissociation kinetics of Fig. 2. k_{off} was calculated directly from the slope; k_{on} was calculated as the difference of the apparent k_{on} determined directly from the slope of the association experiment and k_{off} determined from the dissociation experiment divided by the total ligand concentration. K_d was calculated as k_{off}/k_{on}.

taining versions with 8-N_3-[^{3}H]cAMP and hydrolyzed the electrophoretically purified proteins (see Fig. 1, lanes 11 and 12) with either pronase (to preserve Trp) or HCl. Modified radiolabeled amino acids were identified in parallel by TLE (Figs. 4A and 4B) and by HPLC (Figs. 4C and 4D) by comparison with standard amino acids labeled with the photoreactive analogue. (To protect the primary amino groups from modification in these standards, Trp and Tyr were labeled as Gly–Trp and Gly–Tyr dipeptides and digested with pronase subsequently). When the two anchor-containing proteins were analyzed by these two methods, Trp could be detected as the only radiolabeled residue in pronase-digested material in each case (Figs. 4A to 4D). (Identical results were obtained when vesicles from plasma membranes or mitochondria had been irradiated in the presence of 8-N_3-cAMP, data not shown.) Hydrolysis with HCl (Figs. 4C and 4D) to destroy Trp as well as photoaffinity labeling in the presence of excess nonradioactive cAMP (not shown), both considered as controls, yielded no labeled amino acid at all.

The cytoplasmic cAMP receptor, on the other hand, was very poorly labeled (see also Fig. 1), and the label was not associated with any particular amino acid residue (not shown). This observation is consistent with the absence of both Trp and Tyr from the primary structure of the yeast cytoplasmic R subunit at positions comparable to where these residues are found and become labeled in vertebrate R subunits (6, 31, 32).

Effect of Lipolytic Anchor Cleavage on Photolabeled Amino Acids

To examine the possibility that lipolytic cleavage of the anchors leads to refolding of the cAMP binding domain(s) as the basis of the changes observed in ligand association and dissociation, we analyzed whether the pattern of photolabeled amino acids is changed upon removal of the anchors. The plasma membrane cAMP receptor was purified by affinity and phenyl–Sepharose chromatography, the GPI anchor cleaved by incubation with GPI-PLC from *T. brucei* and the hydrophilic form separated from residual uncleaved material by TX-114 partitioning. In the case of the mitochondrial receptor, the organelles were incubated with PC, ATP, and Ca^{2+} (in order to activate an endogenous PL; cf. 18, 19) and the soluble cAMP-binding protein affinity purified on cAMP–Sepharose. The two soluble proteins were photoaffinity labeled with 8-N_3-[^{3}H]cAMP (presence of detergent as in the above experiment with the anchor-containing proteins), hydrolyzed with pronase, and analyzed in parallel by TLE (Figs. 5A and 5B) and HPLC (Figs.

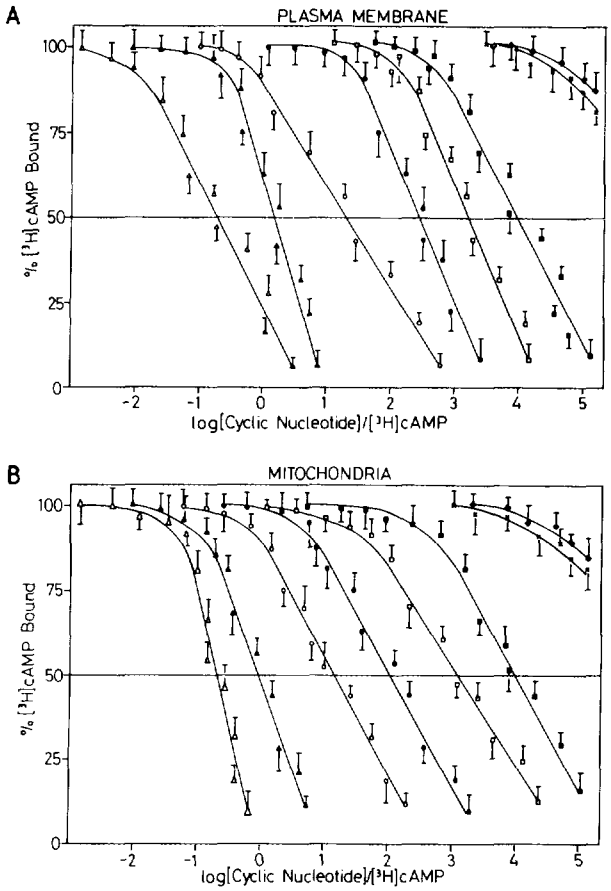

FIG. 3. Competitive inhibition by analogues of [^{3}H]cAMP binding. Solubilized, purified plasma membrane (A) and mitochondria (B). cAMP-binding proteins were incubated with [^{3}H]cAMP in the presence of increasing concentrations of cAMP (▲) and various analogues: △, N^6-[(6-aminohexyl)carbamoyl-methyl]-cAMP; ○, N^6-monobutyryl-cAMP; ●, 8-aminohexylamino-cAMP; □, 8-N_3-cAMP; ■, 8-bromo-cAMP; ×, cIMP; ♦, cGMP. Specific [^{3}H]cAMP-binding in the absence of competitor was set at 100%. The abscissa represents the logarithmic plot of the molar ratios of unlabeled competitor to [^{3}H]cAMP.

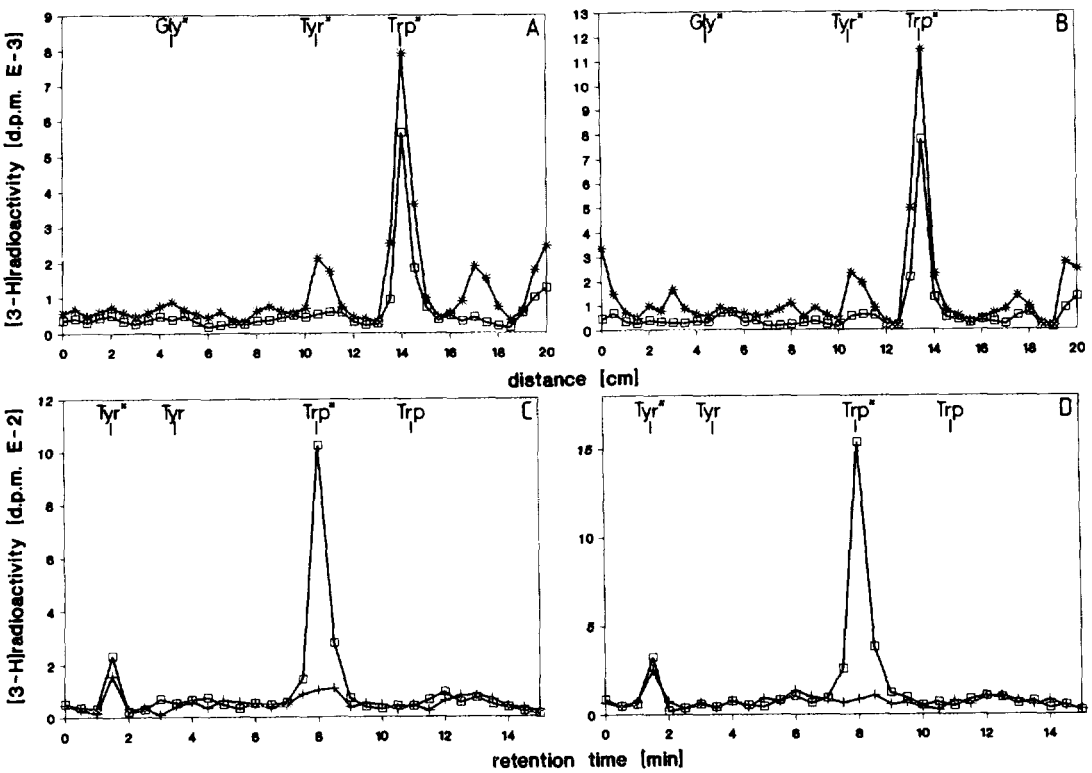

FIG. 4. Determination of the photoaffinity-labeled amino acids in the anchor-containing cAMP receptors. Solubilized, purified plasma membrane (A, C) or mitochondrial (B, D) cAMP-binding proteins were photolabeled with 8-N_3-[^{3}H]cAMP. The radiolabeled proteins were recovered from the detergent phase after TX-114 partitioning, purified by SDS–PAGE, and then digested to completion with pronase (*, □) or hydrolyzed with HCl (+). The radiolabeled amino acids were identified either after two consecutive TLE runs using buffer A [*] or B [□] by a TLC scanner (A, B) or by HPLC (C, D). Origin, cathodic end; front, anodic end; the chromatographic positions of photoaffinity-labeled (Tyr*, Trp*, Gly*) and radiolabeled standard amino acids (Tyr, Trp) are shown on the top.

5C and 5D). In the anchor-free forms of both proteins an additional amino acid is photolabeled as compared to the respective anchor-containing versions (cf. Fig. 4). The second modified amino acid is present in about stoichiometric amounts to Trp and comigrates with photolabeled Tyr in both TLE and HPLC. The nature of the two amino acids was again substantiated by the fact that the peak material, cofractionating with the modified Tyr standard in both TLE and HPLC, was resistant to hydrolysis by HCl, whereas the Trp peak was acid labile.

Analysis of cAMP Binding Centers

In order to corroborate the possibility that two distinct ligand-binding sites are present in the two newly identified membrane-anchored cAMP receptors similarly as in R subunits of PKAs and, if so, to determine which of the two centers is mainly influenced by the cleavage of the anchor, we competed the photomodification by 8-N_3-cAMP with site-selective cAMP analogues (11) and studied the influence of anchor cleavage on the competition. If two different cAMP-binding sites are present in the anchor-containing receptor proteins, it is expected that, in a titration, lower concentrations of competitor are required to replace substoichiometric amounts of radioactive 8-N_3-[^{3}H]cAMP from its preferred binding center, if the respective competitor has the same site selectivity as 8-N_3-cAMP by contrast to the situation where it has the opposite binding preference. We photoaffinity labeled the anchor-containing and the anchor-free forms of the two proteins with substoichiometric amounts of 8-N_3-[^{3}H]cAMP in the presence of increasing concentrations of N^6- and C-8-substituted cAMP analogues which, in mammals, exhibit pronounced binding selectivity for either site A or site B, respectively, and studied the protective effect on the photomodification of either the Trp or the Tyr residue.

Table II compiles the concentrations of analogues required to reduce Trp and Tyr modification by 50% in both forms. In the anchor-containing and anchor-free forms of both proteins, N^6-derivatized cAMP analogues, which preferentially bind to site A of R subunits from mammalian PKAs (see Ref. 11 for binding preferences of analogues in mammalian tissues), are most efficient in protecting Trp from becoming labeled (e.g., N^6-monobutyryl cAMP, cXMP). In this respect the labeled Trp

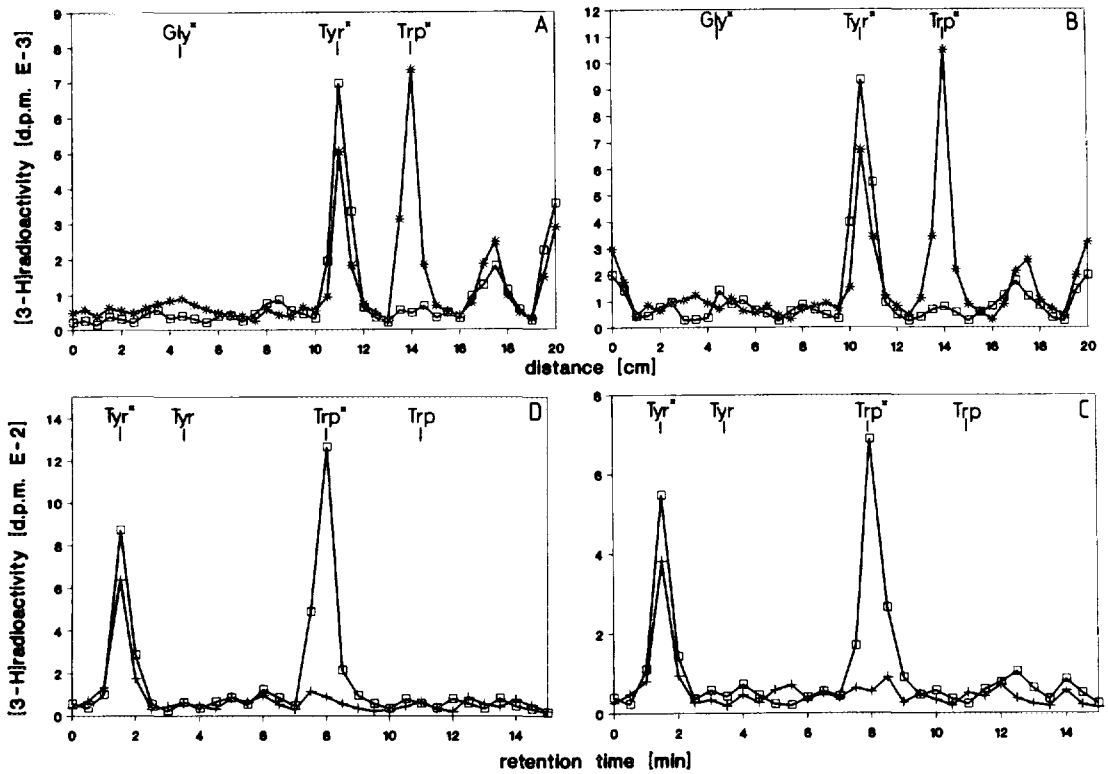

FIG. 5. Determination of the photoaffinity-labeled amino acids in the anchor-free cAMP receptors. The experiments were carried out as described for Fig. 4. After cleavage with GPI-PLC from *T. brucei* (plasma membranes) and PI-PLC from *B. cereus* (mitochondria) and photoaffinity labeling with 8-N_3-[^{3}H]cAMP, the soluble forms were recovered from the aqueous phase after TX-114 partitioning, purified by SDS–PAGE, and then hydrolyzed with pronase (✳) or HCl (□, +). The results of the TLE (first run only) and HPLC analyses are shown.

behaves as expected for a residue in site A of mammalian R subunits. Of unlabeled C-8-modified analogues (e.g., 8-N_3-cAMP and 8-methylamino cAMP), which preferentially bind to site B of both mammalian RI and mammalian RII, significantly higher concentrations are needed to protect Trp. This argues that these ligands preferentially bind to a different center, not containing the Trp residue. This is particularly compelling in the experiment where unlabeled 8-N_3-cAMP is used to compete for the modification of Trp by radiolabeled 8-N_3-cAMP. If only one cAMP-binding site were present, a near to 1:1 stoichiometry would be expected for successful competition. Instead it is observed that the reduction by 50% of Trp modification requires an about 80 to 400 times excess of the unlabeled analogue. This means that competitive inhibition of 8-N_3-[^{3}H]cAMP-binding to the vicinity of Trp occurs only after the saturation of a more affine second site. Consequently, both anchor-modified cAMP-binding proteins must contain two cAMP-binding centers, differing in affinity for the analogue and becoming sequentially saturated in a fashion quite similar to sites A and B of R subunits of PKAs from vertebrate tissues. The Trp residue is close to the center equivalent to site A in mammalian R subunits.

To study the effect of anchor cleavage and to confirm that the Tyr residue additionally amenable to photolabeling after lipolytic removal of the anchor is, in fact, in a separate site, we labeled the soluble proteins with 8-N_3-[^{3}H]cAMP in the presence of site-selective unlabeled analogues as described for the anchor-containing forms. In this case both spots from TLE representing photomodified Trp and Tyr, respectively, were evaluated separately. Table II shows that anchor cleavage has little effect on the analogue concentrations required to protect Trp. On the one hand, of site A-selective N^6-monobutyryl cAMP low concentrations are required to protect Trp, likewise in the anchor-containing and the soluble, anchor-free form, suggesting that cleavage of the anchor leads to only minor changes in the Trp-containing binding center. On the other hand, an about 100-fold excess of the same unlabeled competitor is necessary to inhibit Tyr modification by photoreactive 8-N_3-cAMP by 50%. This suggests that Tyr is in a different site, complementary to the results obtained with the anchor-containing proteins. In contrast to above results with the protection of the Trp residue, unlabeled 8-N_3-cAMP and 8-methylamino cAMP protect Tyr from photoaffinity labeling in close to stoichiometric amounts. These concentrations are considerably lower

TABLE II

Competitive Inhibition by Analogues of Photolabeling of the Anchor-Containing and Free cAMP-Binding Proteins

Origin	cAMP analogue	Molar excess of analogue		
		Trp (anchor-containing)	Trp (anchor-free)	Tyr (anchor-free)
Plasma membranes	*N*(6)-monobutyryl cAMP	9.5	5.7	126.5
	cXMP	12.9	26.8	15.6
	8-N_3-cAMP	415.5	214.8	2.8
	8-Methylamino cAMP	197.2	157.9	4.0
	8-(4-Chlorophenylthio) cAMP	165.4	289.4	167.5
Mitochondria	*N*(6)-monobutyryl cAMP	3.9	5.4	67.8
	cXMP	3.3	4.7	7.7
	8-N_3-cAMP	83.1	69.1	2.1
	8-Methylamino cAMP	33.6	58.7	1.7
	8-(4-Chlorophenylthio) cAMP	71.2	53.1	174.8

Note. Solubilized, purified cAMP-binding proteins were photoaffinity labeled with 8-N_3-[^{3}H]cAMP in the presence of increasing concentrations of various analogues. The anchor-containing and anchor-free cAMP receptors were digested with pronase and then analyzed by TLE. The regions of the plate corresponding to photoaffinity-labeled Trp and Tyr were counted for radioactivity. The radioactivity recovered in the absence of competitor was set at 100%. The values represent molar ratios of unlabeled competitor to 8-N_3-[^{3}H]cAMP required to obtain 50% inhibition of photoaffinity labeling (means of three determinations).

than those required to inhibit modification of the reactive Trp, corroborating that Tyr and Trp are in separate sites. The high concentration of 8-(4-chlorophenylthio)-cAMP which binds preferentially to sites A and B of mammalian type I subunits and the low concentration of cXMP, preferentially selecting sites A and B in RII (11), required to protect simultaneously Trp and Tyr, suggest that the cAMP-binding characteristics of the two membrane-derived receptors from yeast display similarities to RII subunits of mammalian PKAs. Trp is in a center equivalent to site A and Tyr is close to site B.

Interestingly, the distribution of photolabel between Trp and Tyr is only marginally influenced by the specificity and the origin of the PL used for cleavage. The results with GPI-PLD from rabbit serum were qualitatively comparable with those obtained with (G)PI-PLCs from *B. cereus, S. aureus, B. thuringiensis,* and *T. brucei* (Table III).

DISCUSSION

In mammals, several isoforms of R subunits of PKA of both type I and type II exist. They occur both soluble in the cytoplasm and associated with a number of cellular membranes, including plasma membranes. Binding to membranes presumably involves protein/protein interactions exerted between amphipathic helices of R subunits and helix-binding membrane proteins (33). The membrane-bound forms of PKA differ substantially from those described here in that they (i) are associated with their resident membrane from the cytoplasmic side, (ii) lack a lipid-containing membrane anchor, (iii) can be dissociated from the membrane by high salt or carbonate, and (iv) release an active C subunit in the presence of cAMP.

The cAMP-binding proteins described here are particularly distinguished by their membrane anchorage through a (glyco)lipid structure. Recently, we observed that cleavage of the anchor is a physiological process which can be induced by a nutritional upshift and is effected through the glucose-dependent activation of an intrinsic phospholipase C (22a). Here we show that cAMP

TABLE III

Photolabeling of Trp and Tyr in the Anchor-Containing and Free cAMP-Binding Proteins

Origin	Treatment	Trp (dpm)	Tyr (dpm)
Plasma membranes	Octyl glucoside	2601 ± 521	123 ± 41
	GPI-PLD (rabbit serum)	1706 ± 297	1304 ± 189
	PI-PLC *B. cereus*	1563 ± 548	1398 ± 217
	PI-PLC *S. aureus*	1478 ± 214	1631 ± 356
	PI-PLC *B. thuringiensis*	1519 ± 365	1455 ± 423
	PI-PLC *T. brucei*	1230 ± 256	1972 ± 505
Mitochondria	Deoxycholate	3524 ± 716	283 ± 197
	GPI-PLD (rabbit serum)	2641 ± 388	2131 ± 377
	PI-PLC *B. cereus*	2978 ± 478	2650 ± 538
	PI-PLC *S. aureus*	3366 ± 504	2988 ± 497
	PI-PLC *B. thuringiensis*	4165 ± 579	3843 ± 412
	Ca^{2+}/TPA	1978 ± 248	2323 ± 505

Note. Purified cAMP-binding proteins were digested with various phospholipases. Alternatively, mitochondria were incubated with Ca^{2+} plus TPA prior to affinity purification of the cAMP-binding protein. After photoaffinity labeling the hydrophilic proteins were digested with pronase and the photolabeled amino acids analyzed by HLPC and counted for radioactivity. Each value represents the means ± SE of six runs with three independent protein preparations each.

analogue-binding to the two newly identified cAMP receptors is modulated by the presence or absence of the anchors. Both lipid-anchored cAMP receptor proteins contain two cAMP-binding centers, one of which is affected by the lipolytic cleavage. This is evident from the analysis of the aromatic amino acid residues which can be photoaffinity labeled with 8-N_3-cAMP in the anchor-containing and anchor-free cAMP-binding proteins from yeast. In the two PI-anchored cAMP receptors from yeast a single Trp residue can be photolabeled by 8-N_3-cAMP, whereas, after cleavage of the PI-containing membrane anchors by PLs, two aromatic amino acids, Trp and Tyr, are amenable to modification. This observation is consistent with the assumption that lipolytic cleavage of the anchor changes the binding characteristics of cAMP by inducing a conformational change which mainly affects the ligand-binding centers containing the Tyr residue. Most likely refolding occurs in such a way as to bring the Tyr residue into the vicinity of this site in order to stabilize cAMP-binding to this center, e.g., by stacking interactions between π-electrons of the adenine and the phenyl rings. This stabilization could also explain that simultaneously the affinity to cAMP is increased. These observed changes, induced by anchor cleavage, agree with a regulatory role of lipidic membrane anchorage of the two proteins. Previous results with the mitochondrial PI-anchored isoprotein have shown that this is, in fact, the case. The mitochondrial cAMP-binding protein regulates a protein kinase activity in a cAMP-dependent fashion (16–19). It is normally inactive even in the presence of cAMP. Activation of this kinase requires the previous lipolytic cleavage of the PI-anchor of the cAMP-binding protein, in addition to the binding of cAMP (19). (No cAMP-dependent kinase activity has been found associated with plasma membranes so far.) This intriguing inactivity of the lipid-anchored cAMP receptors indicates that the presence of the anchor influences the properties of the protein, presumably the affinity to a putatively associated catalytic subunit. The finding that the affinity for the cAMP ligand and the number and nature of the photoaffinity-labeled amino acids, in fact, is influenced by the presence or absence of the lipidic membrane anchor corroborates this conclusion. Release from the membrane by detergent fails to cause the same effect, and partial denaturation of the cAMP-binding proteins under cleavage conditions appears a less likely explanation, because the affinity to all ligands, which have been examined, is increased upon lipolytic cleavage. Thus, the observed changes are likely to reflect alterations in the tertiary folding structure. In the case of the mitochondrial cAMP receptor, resumption of an alternative folding structure of the protein, in fact, has been demonstrated to occur concomitantly with the removal of the anchor. This conclusion was based on the finding that lipolytic membrane release (by contrast to solubilization by detergent) was accompanied by the alteration of the proteolytic degradation pattern of the native protein (17). These observations provide the first documentation of an allosteric effect of membrane release by PLs on the folding structure of a lipid-anchored protein.

R subunits of mammalian PKAs of type I and type II differ from one another in their labeling patterns by the photoreactive ligand analogue. It has been reported that in type I R subunits both a Trp and a Tyr residue can be crosslinked to 8-N_3-cAMP, whereas in type II two Tyr residues are modified (31, 32). The yeast PKA tentatively has been classified as belonging to type II (3, 12) due to the occurrence of an autophosphorylation site (RRTSV), although the molecular mass of this protein more closely resembles that of mammalian type I R subunits. However, the binding of cAMP analogues has not yet been studied with this protein. We show here that no aromatic amino acids can be photomodified in any of the binding centers of the R subunit of yeast cytoplasmic PKA. This observation substantially contrasts to mammalian subunits of either type, but is in accordance with the absence of aromatic residues from the amino acid sequence of the yeast protein at positions comparable to those photomodified in mammalian R subunits of either type RI or type RII.

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