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In Vitro Effects of Oxygen-derived Free Radicals on Type I and Type II cAMP-Dependent Protein Kinases*

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Oxygen free radicals may act as second messengers in signal transduction pathways and contribute to inflammatory diseases. We studied the action *in vitro* of radiolytically generated hydroxyl radicals (·OH) and superoxide radicals ($O_2^{\cdot-}$) on the cAMP-dependent protein kinases, I and II (PKAI and -II, respectively). The effects of the gasses O_2 and N_2O used to produce $O_2^{\cdot-}$ or ·OH radicals by γ-radiolysis of the water were also studied. PKAI is more sensitive than PKAII to oxygen gas (10 mM sodium formate) and to hydroxyl and superoxide radicals. Hydroxyl radicals decreased the kinase phosphotransferase activities stimulated either by cAMP or its site-specific analogs for both PKAI and PKAII; however, PKAI was more affected. The binding of [^{3}H]cAMP and of 8-N_3-[^{32}P]cAMP to RI regulatory subunits was decreased. ·OH caused a loss of tryptophan 260 fluorescence at site A of PKAI and of bityrosine production. Superoxide radicals affected only PKAI. $O_2^{\cdot-}$ modified both cAMP-binding sites A and B of the regulatory subunit but had a smaller effect on the catalytic subunit. The catalytic subunit was more sensitive to radicals when free than when part of the holoenzymes during exposure to the oxygen free radicals. These results suggest that oxygen free radicals alter the structure of PKA enzymes. Thus, oxidative modifications may alter key enzymes, including cAMP-dependent protein kinases, in certain pathological states.

Reactive oxygen species (ROS)[1] including the superoxide radical ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), and the hydroxyl radical (·OH) are generated in several cell types in response to stimulation by various hormones and cytokines (1–5). The oxygen radicals generated in turn appear to act as second messengers in the activation of the transcription factor NF-κB (5) and in the oxidative modification of several important proteins in various cell types (6–10). H_2O_2 influences the activity of a number of enzymes involved in cellular signaling pathways, including the low k_m cAMP phosphodiesterase (11), the soluble guanylate cyclase (12), the protein kinase C (13, 14), the MAP kinase pathway (15), growth factor receptor tyrosine kinase, and tyrosine phosphatase (16).

The elevation of intracellular levels of cAMP and subsequent activation of cyclic AMP-dependent protein kinases (PKA) are events that contribute to the regulation of a variety of cell functions including gene expression, cellular metabolism, and cell proliferation and differentiation. The inactive PKA holoenzyme is a tetramer consisting of two regulatory (R) subunits and two catalytic (C) subunits. There are two major types of PKA (PKAI and PKAII), which display different biochemical properties due to differences in their R subunits (RI and RII). RI and RII differ in molecular weight, antigenicity, amino acid sequence of the N-terminal domain, ability to be autophosphorylated, and affinity for cAMP analogs. Genetic studies have revealed numerous types of PKA subunit (RIα, RIβ, RIIα, RIIβ, Cα, Cβ, Cγ). PKA is activated by the binding of four cAMP molecules. They bind to two asymmetric sites (designated A and B), on each monomer in a positive cooperative fashion. This results in the dissociation of the holoenzyme to release active C subunit and dimers of the R subunits (for a review see Refs. 17 and 18).

The cAMP-dependent protein kinases in psoriatic cells are abnormal (19). Both 8-N_3-[^{32}P]cAMP binding to the regulatory subunits RI and RII and the cAMP-dependent protein kinase activities are low (20). Indeed, the loss of cAMP binding activity correlates with the severity of the disease (20). This alteration of PKA in psoriatic fibroblasts may be due to oxidative modification (21), and the oxidative state of psoriatic cells might be modified (22–24). Thus, oxidative modifications may modulate PKA activity and be the basis of alteration in key enzyme activities, including PKA, in certain pathological states such as psoriasis.

We present an *in vitro* analysis of the ability of particular oxygen free radicals generated by γ-radiolysis of water to alter the enzymatic properties of PKAI and PKAII. We have also studied the effects of the gasses N_2O and O_2 (in the presence of 10 mM sodium formate) in aqueous solutions that generate 90% hydroxyl radicals (·OH) or 100% anion superoxide radicals ($O_2^{\cdot-}$), respectively.

We report specific alterations in cAMP binding to the regulatory subunits and specific changes in cAMP-dependent protein kinase activities in response to the oxygen free radicals studied.

MATERIALS AND METHODS

Protein Origins—PKA type I was extracted from rabbit skeletal muscle, which contains mostly the RIα isoform (25). PKA type II and C subunit were extracted from bovine heart muscle. PKA type II contains essentially the RIIα isoform (26). All of these products were obtained from Sigma-Aldrich (Saint Quentin Fallavier, France).

Oxygen Radical Generation—Oxygen radical species were homoge-

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[1] The abbreviations used are: ROS, reactive oxygen species; MOPS, 3-(*N*-morpholino)propanesulfonic acid; MES, 4-morpholineethanesulfonic acid; 8-N_3-[^{32}P]cAMP, 8-azidoadenosine 3′:5′-mono[^{32}P]phosphate; 8-AHA-cAMP, 8-aminohexylamino-cAMP; PKA, cAMP-dependent protein kinase; PKAI and PKAII, cAMP-dependent protein kinase I and II, respectively; N^6-Bnz-cAMP, N^6-benzoyl-cAMP; PAGE, polyacrylamide gel electrophoresis.

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neously generated in aqueous protein solutions by γ-radiolysis with a ^{60}Co source (performed at the Laboratoire de Chimie Physique CNRS, URA 400, Université René Descartes, Paris, France). A 1-ml solution of 10 mM-sodium phosphate buffer, pH 7.4, containing 5 μg of the indicated protein was kept cold on ice, saturated for 30 min with the appropriate gas, and then irradiated in closed vessels. The saturation of the solution with N_2O or O_2 gas in the absence or in the presence of 10 mM sodium formate allowed the generation of 90% hydroxyl radicals ($^{\cdot}OH$), or 100% anion superoxide radicals ($O_2^{\cdot-}$), respectively. The doses were delivered at a rate of 2.5 ± 0.1 grays/min, as measured by Fricke's method (27). A single dose of 900 grays was delivered to each protein solution, such that the ratio of oxygen radical to protein (nmol of radicals/nmol of protein) was between 2500 ($^{\cdot}OH$) and 5040 ($O_2^{\cdot-}$), according to the nature of the free radical (in steady-state kinetic conditions).

Protein Kinase Assay—Cyclic AMP-dependent protein kinase activities were measured using kemptide as the phosphate acceptor (a specific substrate for cAMP-dependent protein kinases) (28). One μg of PKAI or PKAII was used to catalyze the transfer of ^{32}P from ATP (5000 pmol; 5×10^5 cpm) to 100 μM of kemptide in the presence of 50 mM MOPS, pH 7, 250 μg/ml bovine serum albumin, 10 mM $MgCl_2$, 100 μM ATP, and the appropriate concentration of cAMP or its analogs (10^{-9} to 10^{-3} M) in a total volume of 50 μl. C subunit activity was determined in the same reaction mixture in the absence of cAMP and its analogs. The phosphorylation reaction was allowed to proceed at 37 °C for 10 min with continuous agitation. The reaction then was terminated by spotting 25 μl of the reaction mixture onto phosphocellulose P-81 strips (Whatman), which were immediately dropped into ice-cold 0.5% phosphoric acid (10 ml/paper strip). The strips were washed three times in 0.5% phosphoric acid with swirling. Radioactivity retained on the P-81 papers was counted in Ready Safe scintillation solvent (Beckman, Fullerton, CA). The background count that was obtained in the absence of enzyme was subtracted from all experimental values. PKA activity was calculated by subtracting the activity measured in the presence of cAMP or its analog from the activity measured in the absence of cAMP.

Antibodies—The polyclonal antibodies against bovine skeletal muscle RIα (25) and against rat heart RIIα (26) were prepared as reported previously. The polyclonal antibody against bovine heart catalytic subunit C was a generous gift from Dr. S. Lohmann (Labor für Klinische Biochemie, Medizinische Universitätsklinik, Würzburg, Germany).

Immunoblotting—Protein kinase preparations (5 μg of PKAI or PKAII or 0.25 μg of catalytic subunit solution) were heated at 100 °C for 5 min in electrophoresis sample buffer and subjected to SDS-PAGE on a 10% gel (minigel). Proteins were transferred electrophoretically to a nitrocellulose membrane (Schleicher and Schuell, Dassel, Germany) using a semidry blotting apparatus (Bio-Rad). The membranes were soaked for 1 h in phosphate-buffered saline containing 0.1% Tween 20 and 5% skim milk. The blots were probed with the specified antibody (anti-RIα (diluted 1:100), anti-RIIα (1:1000), or anti-C (1:500)) by incubation in phosphate-buffered saline containing 0.1% Tween 20 and 5% skim milk overnight at 4 °C and then washed with phosphate-buffered saline containing 0.1% Tween 20 for 30 min. Bound anti-RI antibody was detected by chemiluminescence (ECL, Amersham Pharmacia Biotech, Les Ulis, France) after incubation with the anti-rabbit peroxidase-coupled secondary antibody. Bound anti-RIIα and anti-C antibodies were revealed by a goat anti-rabbit IgG conjugated to alkaline phosphatase (1:7500, Promega, Lyon, France) and NBT and BCIP as substrates.

Photoaffinity Labeling with 8-N_3-[^{32}P]cAMP—RI and RII regulatory subunits were photoaffinity-labeled as described previously (29) in a reaction mixture (80 μl) containing 10 mM MES, pH 6.2, 10 mM $MgCl_2$, 1.0 μM 8-N_3-[^{32}P]cAMP, and 10 μg of PKA. Where indicated, 100 μM cAMP was included to block 8-N_3-[^{32}P]cAMP binding to determine nonspecific labeling. Mixtures were incubated for 60 min in the dark at 4 °C and then irradiated for 10 min with a UV lamp to allow irreversible photoaffinity binding of 8-N_3-[^{32}P]cAMP to the RI and RII subunits. The irradiated samples were pipetted into 20 μl of stop solution (9% SDS, 15% (v/v) glycerol, 6 mM EDTA, 250 mM Tris-HCl, pH 8) and heated at 100 °C for 2 min. Then, 2 μl of 2-mercaptoethanol and 5 μl of 0.1% bromphenol blue in 50% (v/v) glycerol was added, and the samples were electrophoresed in 8.75% polyacrylamide slab gels containing SDS. The gels were dried and autoradiographed at −80 °C using Cronex 4 DuPont medical x-ray film. Band intensities on autoradiograms were quantitated by scanning with a microdensitometer. Ten μg of protein labeled with 8-N_3-[^{32}P]cAMP was applied to each gel lane to allow comparison between the different lanes and autoradiograms. Care was taken not to overexpose the x-ray films. Under these conditions, peak heights obtained by scanning were proportional to the total radioactivity of the corresponding bands as estimated by scintillation counting as described by Walter (30). Labeling of RI and RII regulatory subunits was calculated by integrating the areas under the curves and subtracting nonspecific labeling (in the presence of 0.1 mM cAMP).

Cyclic AMP Binding Assay—Cyclic AMP binding activity was determined by the Millipore filtration method (Ref. 31, as modified in Ref. 32). The incubation mixtures (225 μl) containing buffer A (50 mM Tris-HCl buffer, pH 7.4, 10 mM $MgCl_2$, 6 mM theophylline, 4 mg/ml bovine serum albumin), a standard series of concentrations of [^3H]cAMP (Centre d'Energie Atomique, Saclay, France, 30 Ci/mmol; 0.2–7 pmol) and 10 μg of the different types of PKA were incubated for 2 h at 4 °C. The reaction was terminated by adding 4 ml of buffer A without bovine serum albumin. The samples were immediately loaded onto Millipore filters (0.45 μm, Millipore Corp., HAWPO 2500). The filters were washed twice with 4 ml of buffer A without bovine serum albumin. Radioactivity retained on the filters was determined by counting in Ready Safe scintillation solvent (Beckman, Fullerton, CA). The blank value (nonspecific binding in presence of 0.1 mM cAMP) was subtracted from the total binding value.

Phosphorylation of RII by the Catalytic Subunit of PKA and by Casein Kinase II—RII was phosphorylated by incubation of 2 μg of PKAII holoenzyme in the presence of 5 nM of the exogenous catalytic subunit of PKA in 20 mM MOPS, 20 mM $MgCl_2$, 84 mM Tris-HCl (pH 7.4), 100 μM ATP, and 0.5 μCi of [γ-^{32}P]ATP in a final volume of 15 μl. RII was also phosphorylated by the endogenous catalytic subunit of PKAII in the presence of 0.1 mM cAMP to dissociate the holoenzyme. For RII phosphorylation by casein kinase II, 15 nM of casein kinase II (a generous gift from Claude Cochet, INSERM U.244, Grenoble, France) was added to the incubation reaction, and 50 μM H-89 (Seikagaku Corp., Coger, Paris, France) was also added to inhibit the phosphorylation of RII by the endogenous catalytic subunit. After 10 min at 37 °C, the reaction was terminated by the addition of 5 μl of stop solution (200 mM Tris-HCl, pH 6.8, 8% SDS, 20% glycerol, 5% 2-mercaptoethanol, and 0.01% bromphenol blue) and heating at 100 °C for 5 min. Samples were then processed for SDS-polyacrylamide gel electrophoresis and autoradiography.

Tryptophan, Tyrosine, and Bityrosine Fluorescence—Fluorescence was measured at 23 °C using 100 μl of 0.1 μM PKAI and PKAII in a quartz cuvette in a Perkin-Elmer spectrofluorimeter. For tryptophan fluorescence, the excitation wavelengh was 290 nm with a bandpass of 10 nm. The excited samples were scanned from 300 to 450 nm using an emission bandpass of 2.5 nm. For tyrosine fluorescence, the excitation wavelengh was 280 nm with a bandpass of 2.5 nm, and the excited samples were scanned from 300 to 400 nm using an emission band of 2.5 nm. For bityrosine fluorescence, the excitation wavelength was 325 nm with a bandpass of 2.5 nm, and the excited samples were scanned from 360 to 470 nm using an emission bandpass of 4 nm. Fluorescence was titrated three times independently. For cAMP titrations, PKAI was incubated with each of a series of concentrations of cAMP (0–100 μM) (33). The excitation and emission wavelengths were 295 and 341 nm, respectively.

Densitometric Scanning of Autoradiograms—Bands on autoradiograms from nonsaturated autoradiography of Western blots were scanned densitometrically. The results are expressed as relative optical density units. The peak heights obtained by scanning were proportional to the amount of protein loaded onto the gel, and values were corrected according to the variations in the controls.

RESULTS

The Gasses (O_2 or N_2O) and Superoxide and Hydroxyl Radicals Have Different Effects on the Phosphotransferase Activity of cAMP-dependent Protein Kinases PKAI and PKAII—To produce superoxide or hydroxyl radicals selectively during H_2O radiolysis with γ-irradiation, aqueous solutions of PKA were saturated either with O_2 (in the presence of sodium formate) or N_2O alone. The phosphotransferase activities of rabbit skeletal muscle PKAI and of bovine heart muscle PKAII holoenzymes exposed to these oxygen free radical-generating systems were measured as the phosphorylation of a specific substrate in the presence and absence of cAMP. The various conditions of free radical generation did not alter the basal transferase activities of PKA in the absence of cAMP. However, the basal phosphotransferase activity of PKAI saturated with O_2 (in the presence of sodium formate) was higher than that in controls.

Incubation of PKAI in water saturated with O_2 (in the presence of sodium formate) caused a 50% decrease in the V_{max} of

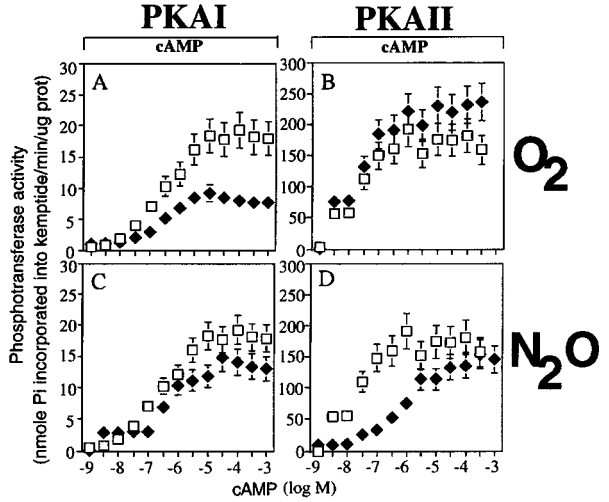

FIG. 1. ***In vitro* effect on purified PKAI and PKAII of saturation with O_2 (*A* and *B*) and N_2O (*C* and *D*).** Purified preparations of rabbit PKAI (*A* and *C*) and bovine PKAII (*B* and *D*) were incubated under control conditions (*hollow squares*) or under conditions of saturation with O_2 gas in the presence of 10 mM sodium formate or with N_2O in closed vessels (*solid diamonds*). Phosphotransferase activities were measured at various concentrations of cAMP. Results of kemptide phosphorylation are expressed as nmol of inorganic phosphate incorporated per min per μg of protein. Results are the mean of three experiments.

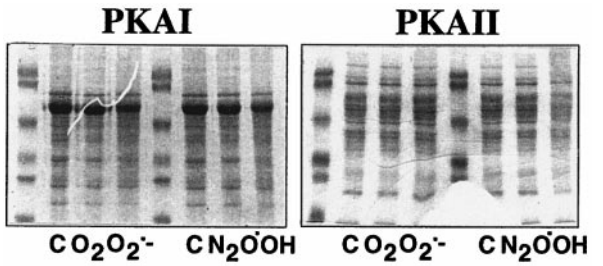

FIG. 2. **The pattern of Coomassie Blue staining of the PKA proteins after saturation of gasses and γ-irradiation.** *Lanes 1* and *4* in each *panel* are the molecular weight standards.

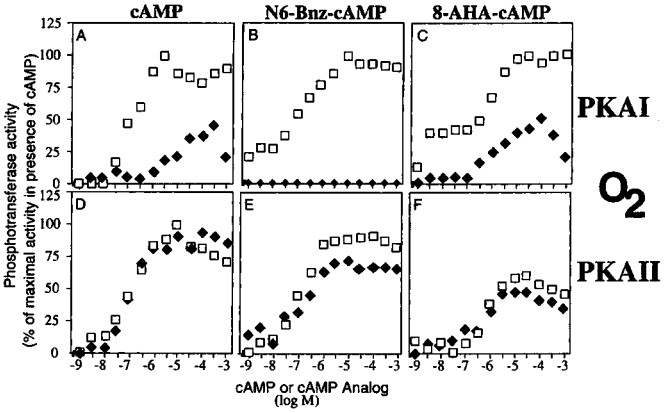

FIG. 3. ***In vitro* effect of superoxide radicals generated by γ-radiation on purified rabbit PKAI and bovine PKAII.** Preparations of PKA were saturated with O_2 in the presence of 10 mM sodium formate in closed vessels without irradiation (control, *hollow squares*) or with irradiation to generate $O_2^{\cdot-}$ (*solid diamonds*). Phosphotransferase activities of control and irradiated PKAI (*A–C*) and PKAII (*panels D–F*) were determined at various concentrations of cAMP (*A* and *D*) and of two cAMP analogs: (N^6-Bnz-cAMP (*B* and *E*), which selectively binds to site A of RI and RII, and 8-AHA-cAMP (*AHA-cAMP*; *C* and *F*), which selectively binds to site B of both RI and RII. Results of kemptide phosphorylation are expressed as the percentage of maximal activity in the presence of cAMP. The maximal value of cAMP-dependent kinase activity obtained in presence of the O_2 without irradiation was used as the 100% value. Results are the mean of two experiments.

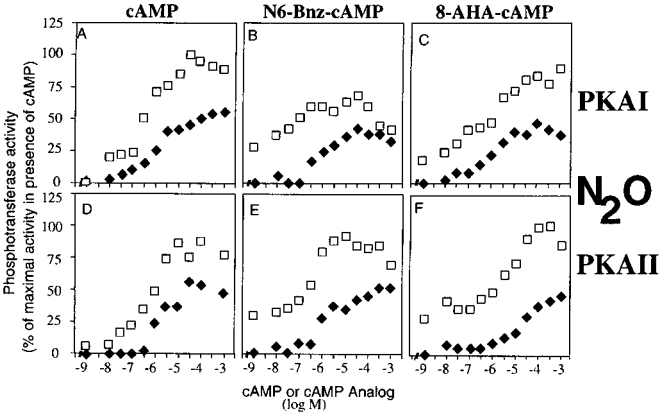

FIG. 4. ***In vitro* effects of hydroxyl radicals generated by γ-radiation on purified rabbit PKAI and bovine PKAII.** Preparations of PKA were saturated with N_2O in closed vessels without irradiation (*hollow squares*, *A–C* for PKAI; *D–F* for PKAII) or with γ-radiation to generate $^{\cdot}OH$ (*solid diamonds*; *A* and *D*). See the legend to Fig. 2 for further details. The maximal value of cAMP-dependent kinase activity obtained in the presence of N_2O without irradiation was used as the 100% value. Results are the mean of two experiments.

the cAMP-dependent phosphotransferase activity without modification of the K_{act} (*i.e.* the concentration of cAMP required for half-maximal kinase activation; control/O_2 = 3×10^{-7} M) (Fig. 1*A*). In contrast, the cAMP-dependent phosphotransferase activity of PKAII was increased by the same conditions (Fig. 1*B*). A decrease in cAMP-dependent phosphotransferase activity of both type I and type II PKA was observed after incubation in the presence of N_2O (Fig. 1, *C* and *D*), and the K_{act} of cAMP for PKAII but not that for PKAI was modified (for PKAII, $K_{act(C)} = 3 \times 10^{-8}$ M/$K_{act(N_2O)} = 3 \times 10^{-7}$ M).

We determined the effects of a series of doses of radiation on the cAMP-dependent activities of PKAII. The effect of radiation was dose-dependent. The maximal effect on the cAMP-dependent phosphotransferase activity of PKAII was observed with 900 grays in the presence of 50% of superoxide and 50% of hydroxyl anions (21). This dose was used for all of the following studies. Under these experimental conditions, incubation of the holoenzymes in aqueous solutions saturated with O_2 (in the presence of sodium formate) or N_2O and irradiated to produce the ROS ($O_2^{\cdot-}$ or $^{\cdot}OH$), respectively, the protein patterns of the holoenzymes were not noticeably modified as ascertained by Coomassie Blue staining after SDS-PAGE (Fig. 2).

The carboxyl termini of the RI and RII regulatory subunits of PKAI and PKAII contain two tandem homologous cAMP binding domains, called sites A and B. These two cAMP binding sites have different specificities for cAMP analogs. Site A has a faster off-rate and a preference for N^6-substituted analogs, whereas site B has a slower off-rate and exhibits a preference for C-2- and C-8-substituted cAMP analogs. We investigated whether these two cAMP binding sites are differentially altered by ROS. The phosphotransferase activities of PKAI and PKAII both before and after irradiation were measured in the presence of each of a series of concentrations of cAMP or its analogs, the N-6-substituted N^6-Bnz-cAMP and the C-8-substituted 8-AHA-cAMP (see "Materials and Methods"; Figs. 3 and 4). The maximum activity of cAMP-dependent kinase obtained in the presence of gas but without irradiation was taken as the 100% value.

Superoxide radical treatment of PKAI caused a 55% decrease in stimulation of PKAI phosphotransferase activity by both cAMP and 8-AHA-cAMP (Fig. 3, *A* and *C*). The apparent activation constant K_{act} was increased (from $K_{act(O_2)} = 3 \times 10^{-7}$ M/$K_{act(O_{2\text{-irr}})} = 10^{-6}/3 \times 10^{-5}$ M). Interestingly, the ability of the A site-selective analog N^6-Bnz-cAMP to activate PKAI was

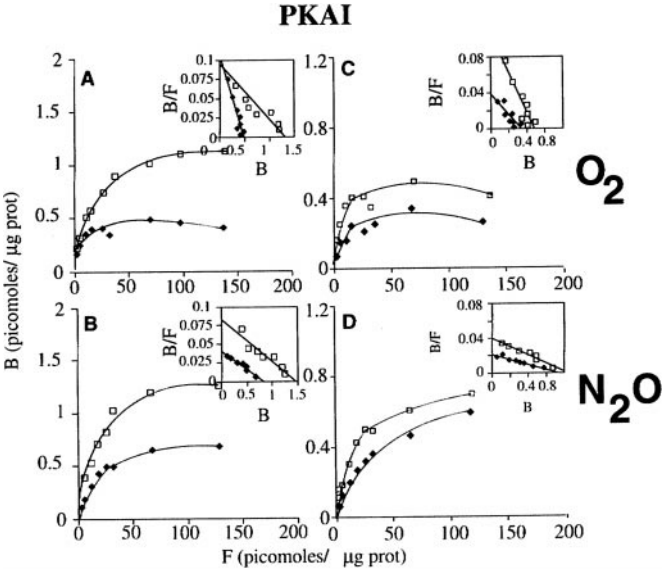

FIG. 5. **Effects of ROS on the saturation curves of specific [^{3}H]cAMP binding to RI from purified rabbit PKAI.** Preparations of PKAI were incubated under conditions of saturation with O_2 gas in the presence of 10 mM sodium formate in closed vessels without irradiation (*solid diamonds*) and PKAI control incubated in air (*hollow squares*; *A*). *C*, preparations of PKAI were incubated under saturation conditions with O_2 in the presence of 10 mM sodium formate without (*hollow squares*) and with (*solid diamonds*) γ-radiation. *B*, preparations of PKAI were incubated in air control (*hollow squares*) and under conditions of saturation with N_2O (*solid diamond*). Preparations of PKAI were saturated with N_2O in closed vessels with irradiation (*solid diamonds*) or without irradiation (*hollow squares*; *D*). The treated PKAI (10 µg of protein) preparations were incubated for 2 h at 4 °C with various concentrations of [^{3}H]cAMP in the absence (total binding) or presence (nonspecific binding) of 0.1 mM unlabeled cAMP. Specific binding was calculated by subtracting nonspecific from total binding. Scatchard plots derived from each saturation curve are shown in the *insets*.

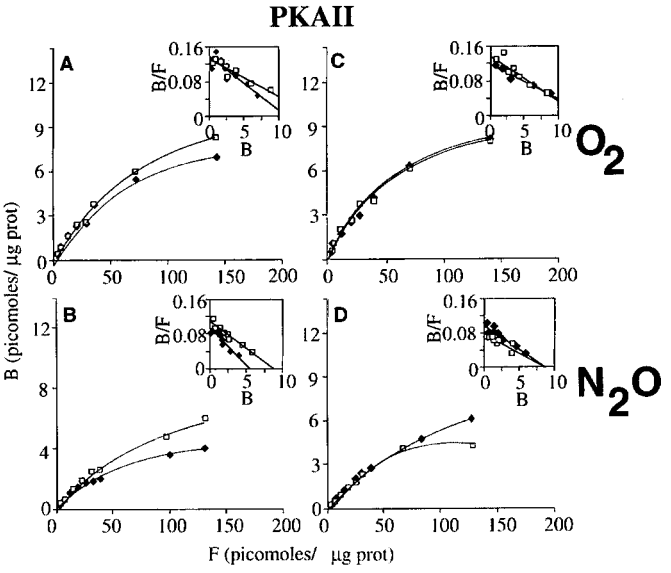

FIG. 6. **Effects of ROS on the saturation curves of specific [^{3}H]cAMP binding to RII from purified bovine PKAII.** The PKAII solutions (*A* and *B*, respectively) were saturated with O_2 in the presence of 10 mM sodium formate in closed vessels and irradiated (*solid diamonds*) or not (*hollow squares*). Solutions of PKAI and PKAII (*C* and *D*, respectively) were saturated with N_2O in closed vessels and irradiated (*solid diamonds*) or not (*hollow squares*). PKAI and PKAII (10 µg of protein) were incubated for 2 h at 4 °C with various amounts of [^{3}H]cAMP in the absence (total binding) or in the presence (nonspecific binding) of 0.1 mM unlabeled cAMP. Specific binding was calculated by subtracting nonspecific from total binding. The Scatchard plots derived from each saturation curve are shown in the *insets*.

completely abolished by exposure to superoxide radicals (Fig. 3*B*). In contrast, exposure of PKAII to superoxide radicals only slightly reduced stimulation of phosphotransterase activity by cAMP and the cAMP analogs (Fig. 3, *D–F*).

Exposure of PKAI to hydroxyl radicals also resulted in a significant increase in phosphotransferase activity in the presence of cAMP (Fig. 4*A*), N^6-Bnz-cAMP (Fig. 4*B*), and 8-AHA-cAMP (Fig. 4*C*). Exposure of PKAII to hydroxyl radicals had similar effects to those on PKAI: a significant decrease in phosphotransferase activities (Fig. 4, *D–F*). The K_{act} values were also increased under these conditions, most substantially for PKAI activation by N^6-Bnz-cAMP ($K_{act(O_2)} = 10^{-8}$ M/$K_{act(O_{2\text{-irr}})} = 3 \times 10^{-7}$ M).

Effects of Gasses and ROS on cAMP Binding to PKAI and PKAII—The effects of ROS on cAMP binding to the RI and RII regulatory subunits of PKAI and PKAII were determined by Millipore filtration assays with [^{3}H]cAMP or affinity labeling with 8-N_3-[^{32}P]cAMP. The first of these techniques only allows determination of affinity and binding capacity of site B. 8-N_3-[^{32}P]cAMP binds covalently to both sites A and B on RI but only to site B on RII. We also tried to estimate protein-bound [^{3}H]cAMP by ammonium sulfate precipitation according to the method of Ogreid and Doskeland (34), but we were unable to discriminate between sites A and B.

^{3}H-Labeled cyclic AMP binding to the regulatory subunits of PKAI was determined in the presence of oxygen (sodium formate) without irradiation (Fig. 5*A*, *black diamonds*), in the presence of N_2O alone (Fig. 5*B*, *black diamonds*), and after exposure to superoxide (Fig. 4*C*, *black diamonds*) or hydroxyl radicals (Fig. 5*D*, *black diamonds*). The same experiment was performed with PKAII (Fig. 6, *A–D*). The presence of the different gasses did not affect the K_d of PKAII for cAMP binding ($K_{d(C)} = K_{d(O_2)} = K_{d(N_2O)} = 3.6 \times 10^{-6}$ M; Fig. 6, *A* and *B*; Table I). However, incubation of PKAI in the presence of O_2 modified cAMP binding to RI ($K_{d(C)} = 6.2 \times 10^{-7}$ M, $K_{d(O_2)} = 1.8 \times 10^{-7}$ M; Fig. 5*A*, Table I). Incubation of PKAI in the presence of N_2O resulted in decreased cAMP binding to RI ($K_{d(N_2O)} = 9.5 \times 10^{-7}$ M; Fig. 5*B*).

The binding of [^{3}H]cAMP to PKAI was reduced by treatment with superoxide radicals (Fig. 5*C*). There was no change in the binding capacity under these conditions, but a difference in the affinity of RI for cAMP was observed ($K_{d(O_{2\text{-irr}})} = 3.6\ 10^{-7}$ M, $K_{d(O_2)} = 1.8 \times 10^{-7}$ M; Fig. 5*C*). There was no significant difference between cAMP binding to RII in the presence and absence of superoxide radicals (Fig. 6*C*).

Hydroxyl radicals modified [^{3}H]cAMP binding to RI only (Fig. 5*D*). Exposure of PKAI to hydroxyl radicals only affected affinity for [^{3}H]cAMP ($K_{d(N_2O_{\text{-irr}})} = 13.5 \times 10^{-7}$ M (RI), $K_{d(N_2O)} = 9.5 \times 10^{-7}$ M). Treatment of PKAII with hydroxyl radicals resulted in altered binding capacity for [^{3}H]cAMP, although the affinity was not substantially changed ($K_{d(N_2O_{\text{-irr}})} = 2.4 \times 10^{-6}$ M, $K_{d(N_2O)} = 3.3 \times 10^{-6}$ M). RII bound 10 times more [^{3}H]cAMP than RI, as has been previously reported. The K_d values of [^{3}H]cAMP binding for PKAI and PKAII and K_{act} values of cAMP transferase activity are shown in Table I.

Photoaffinity labeling with 8-N_3-[^{32}P]cAMP gave different results with RI and RII. Two amino acid residues are affinity-labeled in RI (Trp^{260} in site A and Tyr^{371} in site B of RI) (35). The ligand binds covalently to the RII subunit, which is modified at a single residue (Tyr^{381} in site B). Thus, the ligand does not bind covalently to the A binding site of RII (35). Tyrosine and tryptophan residues are potential targets for oxidative free radicals (36–37). RI and RII in PKAI and PKAII holoenzymes were photoaffinity-labeled with 8-N_3-[^{32}P]cAMP in the presence of O_2 or N_2O gasses and after exposure to either superox-

TABLE I
The K_d values of [^{3}H]cAMP binding for PKAI and PKAII and K *values of cAMP transferase activity in various conditions*

	K_{act} of cAMP				
	C	O_2	O_2 irr.[a]	N_2O	N_2O irr.
			M		
PKAI	3×10^{-7}	3×10^{-7}	$10^{-6}/3 \times 10^{-5}$	3×10^{-6}	3×10^{-6}
PKAII	3×10^{-8}	3×10^{-7}	10^{-7}	3×10^{-7}	10^{-6}
	K_d of [^{3}H]cAMP				
	C	O_2	O_2 irr.	N_2O	N_2O irr.
			M		
PKAI	6.2×10^{-7}	1.8×10^{-7}	3.6×10^{-7}	9.5×10^{-7}	13.5×10^{-7}
PKAII	3.6×10^{-6}	4×10^{-6}	2.7×10^{-6}	3.3×10^{-6}	2.4×10^{-6}

[a] irr., irradiated.

ide or hydroxyl radicals (Fig. 6, RI and RII). The addition of cAMP competed with the binding of the cAMP analog, evidence of the specificity of photoaffinity labeling (Fig. 6, RI, *lane 2*; RII, *lane 2*). PKAI gave one band (49 kDa) corresponding to RI and a second (37 kDa) corresponding to a proteolytic product. PKAII gave two bands corresponding to the two forms of RII (unphosphorylated RII (54 kDa) and RII phosphorylated by the catalytic subunit of PKA (57 kDa)), and a 46-kDa polypeptide that is a proteolytic product.

Incubation in the presence of O_2 gas (sodium formate) resulted in no appreciable change in the binding of 8-N_3-[^{32}P]cAMP to the RI and RII regulatory subunits as determined by densitometric scanning of the gels (data not shown). Exposure to N_2O gas caused a 30% decrease in the binding of the cAMP analog to RI but not to RII (Fig. 7, compare *lane 1* with *lane 5* for RI and RII).

Treatment of RI with superoxide radicals resulted in a 24% decrease in photoaffinity labeling of RI with 8-N_3-[^{32}P]cAMP (Fig. 7, RI, compare *lane 3* with *lane 4*). Similar exposure of PKAII to superoxide radicals did not modify the covalent labeling of RII (Fig. 7 RII, compare *lane 3* with *lane 4*).

Thus, exposure to hydroxyl radicals greatly reduced 8-N_3-[^{32}P]cAMP labeling of RI but only slightly reduced binding to RII (Fig. 7, RI and RII, compare *lane 5* with *lane 6*). The reduction observed for PKAI was double that for PKAII (78 and 32%).

Effects of ROS on the Immunoreactivity of RI, RII, and C subunits of PKAI and PKAII—Antibodies directed against RIα and RIIα (which are the R isoforms present in the PKA preparations from rabbit skeletal muscle and bovine heart muscle, respectively) and against the C subunit were used to test for modifications in the regulatory subunits RI and RII, and in the catalytic subunit C of the two PKA holoenzymes.

The immunoreactivity of RI, RII, and C subunits was not significantly changed by incubation in the presence of O_2 gas or N_2O gas alone (Fig. 8, *A–D*, *lanes 1*, *2*, and *4*). Treatment of PKAI and PKAII with superoxide radicals did not alter either the immunoreactivity or amounts of RI and RII (Fig. 8, *A* and *B*, *lanes 2* and *3*). Exposure of PKAII to hydroxyl radicals did not result in any apparent loss of RII (Fig. 8*B*, *lanes 4* and *5*). However, exposure of PKAI to hydroxyl radicals induced a significant loss in RI immunoreactivity (Fig. 8*A*, *lane 5*). Incubation with hydroxyl radicals also altered antibody recognition of the catalytic subunit of PKAI (Fig. 8*C*, *lane 5*). The C subunits in PKAI and PKAII were similarly immunoreactive in all other treatment conditions and controls (Fig. 8, *C*, *lanes 1–4,* and *D*, *lanes 1–5*).

Effects of O_2, N_2O Gasses, and ROS on the Phosphorylation of Bovine RII—Bovine heart RIIα is phosphorylated *in vivo* and *in vitro* by the catalytic subunit of PKA (38), by glycogen synthase kinase 3 (39), by casein kinase II (40), and by Cdc2

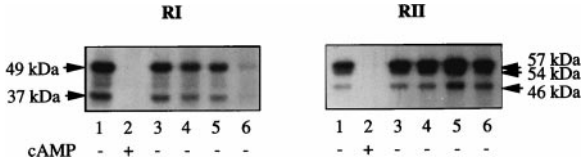

FIG. 7. **Effect of superoxide radicals and hydroxyl radicals generated by γ-radiation on the specific binding of 8-N_3-[^{32}P]cAMP to RI and RII.** Purified preparations of PKAI (RI) and PKAII (RII) were subjected to bubbling with O_2 in the presence of 10 mM sodium formate (*lanes 3*) and irradiation to generate O_2^- (*lanes 4*), or bubbling with N_2O (*lanes 5*) and irradiation to generate $^{\cdot}OH$ (*lanes 6*). Control PKAI and PKAII solutions were incubated without gas (*lane 1* in both *panels*). *Lane 2*, nonspecific labeling in the presence of 0.1 mM cAMP. Ten μg of the PKA regulatory subunit from each sample (PKAI and PKAII) was photoaffinity-labeled with 0.1 μM 8-N_3-[^{32}P]cAMP, separated by 8.75% SDS-PAGE, and subjected to autoradiography. *Arrows* in the *RI* and *RII*, panels, respectively, show RI and RII in the PKAI and PKAII purified preparations.

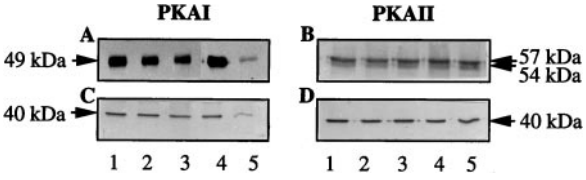

FIG. 8. **Immunoblot analysis of RI, RII, and C subunits.** Preparations of PKAI and PKAII were subjected to selective bubbling with O_2 in the presence of 10 mM sodium formate (*lanes 2*) and irradiation to generate O_2^- (*lanes 3*) or bubbling with N_2O (*lanes 4*) and irradiation to generate $^{\cdot}OH$ (*lanes 5*). Control PKAI and PKAII samples were incubated without gas. Five μg of each protein preparation then were separated by 10% SDS-PAGE and probed with anti-RI (*A*) or anti-C (*C*) for PKAI and anti-RII (*B*) or anti-C (*D*) for PKAII by Western blotting.

kinase (41). The phosphorylation of bovine RII (54 kDa) by the catalytic subunit causes a shift in its electrophoretic mobility to 57 kDa, whereas phosphorylation by the other kinases does not cause this shift.

The ability of the exogenous purified catalytic subunit of heart bovine (Fig. 9*A*) and casein kinase II (Fig. 9*B*) to phosphorylate RII was determined after incubation of PKAII in the presence of O_2 or N_2O gas and after exposure of PKAII to superoxide and hydroxyl radicals. The phosphorylation of RII by the catalytic subunit of PKA and by casein kinase II was decreased by 65 and 26% from control values, respectively, when PKAII holoenzyme was exposed to oxygen in presence of sodium formate (Fig. 9*A*, *lanes 1* and *2*; and Fig. 9*B*, *lanes 1* and *2*). Incubation with N_2O gas alone decreased the phosphorylation of RII by the catalytic subunit of PKAII by about 19% (Fig. 9*A*, compare *lane 4* with *lane 1*). Phosphorylation of RII by each PKA or casein kinase II was similar after exposure to superoxide radicals and O_2 in the presence of sodium formate (Fig. 9, *A* and *B*, *lanes 2* and *3*). In contrast, treatment with hydroxyl radicals strongly inhibited the phosphorylation of RII (60%) by both PKA and casein kinase II (Fig. 9, *A* and *B*; compare *lane 5* with *lane 4*). Similar results were obtained with intraphosphorylation of the RII regulatory subunits by the endogenous catalytic subunits of PKAII.

Effects of O_2, N_2O, and ROS on the Phosphotransferase Activity of the Free Catalytic Subunit of PKAII—The effects of gas and irradiation on the phosphotransferase activity of the free catalytic subunit of PKA were determined (Fig. 10). This activity decreased even after incubation only in gas (Fig. 9, compare *lanes 1* and *2* (55%) and *lanes 1* and *4* (50%)). Exposure to superoxide radicals further decreased the phosphotransferase activity (Fig. 10, *lanes 2* and *3*). This loss in activity was not due to protein degradation as ascertained by silver staining of the gel and immunoblotting of the C subunits with anti-C antibodies after irradiation (data not shown). Treatment with hydroxyl

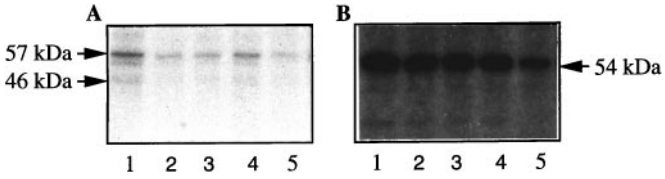

FIG. 9. **Effect of ROS on *in vitro* phosphorylation of RII by its endogenous catalytic subunit and by casein kinase II.** Lanes 1–5, untreated control PKAII incubated in air, PKAII subjected to bubbling with O_2 in the presence of 10 mM sodium formate, irradiation to generate O_2^- or bubbling with N_2O, and irradiation to generate $^{\cdot}OH$, respectively. RII was phosphorylated by the endogenous catalytic subunit (*A*) in the presence of 0.1 mM cAMP as described under "Materials and Methods." Casein kinase II (15 nM) was as used to determine the phosphorylation of RII by casein kinase II (*B*). After incubation for 10 min at 37 °C, the reaction was stopped with Laemmli SDS sample buffer, and the proteins were resolved by 10% SDS-PAGE and detected by autoradiography. *Arrow*, RII.

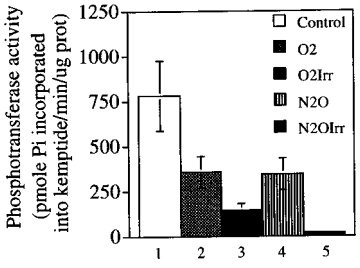

FIG. 10. **Effect of ROS on the phosphotransferase activity of free bovine PKA catalytic subunit (not in the holoenzyme).** *Lanes 1–5* correspond, respectively, to the control C subunit incubated in air (control), bubbling with O_2 in the presence of 10 mM sodium formate (*O2*), irradiation to generate O_2^- (*O_2Irr*), bubbling with N_2O (*N2O*), and irradiation to generate $^{\cdot}OH$ (*N2OIrr*). Phosphotransferase activity was determined as described under "Materials and Methods."

radicals also caused an additional decrease of 95% in the kinase activity.

Loss of Tryptophan and Tyrosine Residues and Bityrosine Production upon ROS Treatment of PKAI and PKAII Holoenzymes—Changes in tryptophan, tyrosine, and bityrosine fluorescence due to exposure to ROS were determined (Fig. 11). There were no significant changes in tryptophan or tyrosine fluorescence (Fig. 11, *A* and *C*) when the PKAI solution was saturated with O_2 (sodium formate), and there was no production of bityrosine (Fig. 11*E*). Incubation of PKAI in the presence of O_2^- decreased the tryptophan and tyrosine fluorescence by 28% (Fig. 11, *A* and *C*) and caused the appearance of a small peak of bityrosine fluorescence (*E*). For PKAII, no loss of tryptophan or tyrosine residues and also no bityrosine production were detected in the presence of O_2 (sodium formate) with or without γ-irradiation (data not shown).

Surprisingly, N_2O gas caused a 25% decrease in the tryptophan fluorescence of PKAI (Fig. 11*B*) but had no appreciable effect on tyrosine fluorescence (Fig. 11*D*) or bityrosine fluorescence (*F*). Hydroxyl radicals significantly reduced tryptophan fluorescence (25%) and tyrosine fluorescence (56%) (Fig. 11*D*) and increased bityrosine fluorescence (Fig. 11*F*). The only condition that reduced the tyrosine signal of PKAII by more than 50% was irradiation with hydroxyl radicals (data not shown).

The intrinsic tryptophan fluorescence of the RI subunit is quenched by cAMP (33). Since the B site of RI does not contain a tryptophan residue, the change in total tryptophan fluorescence of the regulatory subunit in response to cAMP indicates cAMP binding to the A site. The tryptophan fluorescence of the holoenzyme PKAI control was measured. It was partially quenched by the addition of cAMP. The tryptophan fluorescence quenching after saturation in gasses was the same as for controls. A significant decrease in the cAMP quenching of RI was observed after irradiation to generate superoxide and hydroxyl radicals (67 and 75%, respectively).

DISCUSSION

Oxygen free radicals may play a significant role in cell proliferation, differentiation, and the action of certain hormones by regulating transmembrane signaling pathways (5, 13, 16, 42). The amino acid residues tryptophan, tyrosine, histidine, and cysteine are more vulnerable than most other amino acids to modification by free radicals and are potential targets for oxygen free radicals (36, 37, 43). The particular features of PKA need to be recalled to understand the effects of oxygen free radicals on amino acid residues present in the primary structure of these enzymes. The regulatory subunits contain an amino-terminal dimerization domain, the hinge region for interaction with the catalytic subunit, and in the carboxyl terminus two tandem cAMP-binding sites termed site A and site B (44–47). RI subunit dimerization involves two disulfide bridges giving the two RI protomers an antiparallel alignment (46), which may increase the accessibility of the PKAI to free radicals such that it is greater than for PKAII. Dimerization of the RII subunit does not involve cysteine residues. RII can be phosphorylated by several kinases (38, 39), whereas no phosphorylation of RI has been reported. The conserved aromatic amino acids, tryptophan (Trp^{260}) in site A of PKAI and tyrosine near the B cAMP-binding site (Tyr^{371} in RI and Tyr^{381} in RII) are involved in the interaction with the cAMP. Two amino acid residues in RI can be affinity-labeled by cAMP analogs (Trp^{260} in site A and Tyr^{371} in site B of RI; Ref. 34), whereas the RII subunit is modified at only a single residue (Tyr^{381} in site B).

Effects of Gasses on cAMP-dependent Protein Kinases—We first propose to discuss the effects of both gasses (O_2 in the presence of sodium formate and N_2O) on PKAI, then on PKAII holoenzymes, and finally on the free catalytic subunit of these enzymes. Gasses had different effects on the PKAI and PKAII.

Oxygen gas halved the cAMP phosphotransferase activity of PKAI. Also oxygen gas was accountable for an additional 25% dissociation of PKAI in the basal conditions (without cAMP). As attested by the [^{3}H]cAMP binding on site B of RI, there was only one-third the number of binding sites. The quenching of tryptophan fluorescence by cAMP, a marker of site A (Trp^{260}) in RI (33) was also decreased.

N_2O gas had less effect than O_2 gas on PKAI. A 25% decrease of V_{max} of the cAMP phosphotransferase activity of PKAI and a 2-fold decrease of cAMP binding to site B were observed. The total tryptophan fluorescence of PKAI was also reduced, and in particular the quenching of Trp^{260} fluorescence of site A by cAMP was decreased by 17%. These results suggested that N_2O induced a modification in both sites A and B of PKAI.

In contrast, the gasses had less pronounced effects on PKAII than on PKAI. After oxygen saturation, PKAII was not dissociated in the basal condition. PKAII displayed an increase in cAMP-dependent phosphotransferase activity that was O_2-dependent; the O_2 concentration was 2×10^{-4} M in the control PKAII solution and 10^{-3} M after O_2 saturation. Moreover, the RII phosphorylation by several kinases including casein kinase II or the PKA itself was affected. It is likely that the three-dimensional conformation of RII was probably modified by O_2 at the N terminus and at the hinge region.

PKAII was less sensitive to N_2O, which caused an increase in the apparent K_{act} for cAMP ($K_{act(N_2O)} = 3 \times 10^{-6}$/$K_{act(C)} = 3 \times 10^{-7}$ M) and a reduced phosphorylation of RII by the C subunit of PKA. However, there the effects were weaker than those produced by oxygen. Neither O_2 or N_2O under saturation conditions had any effect on cAMP-binding to PKAII, or on the photoaffinity labeling of RI and RII by 8-N_3-[^{32}P]cAMP.

The free phosphotransferase activity of the free catalytic

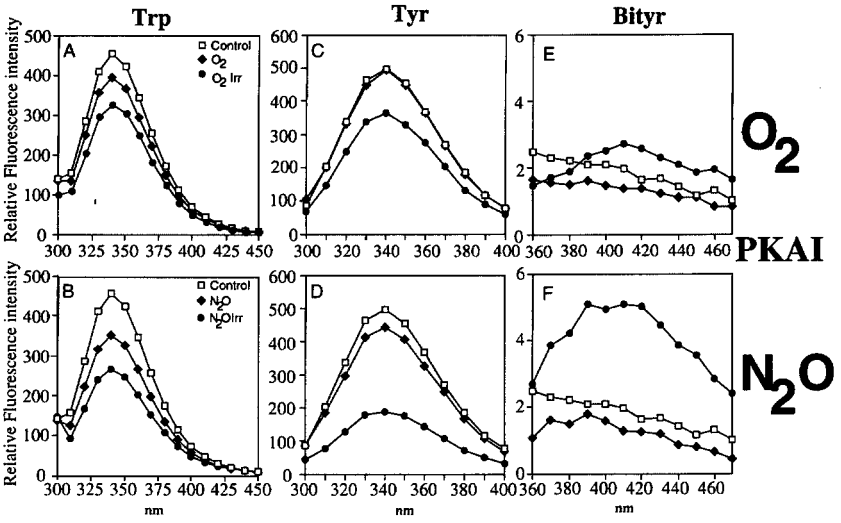

FIG. 11. **Effects of gasses and ROS treatment of PKAI on tryptophan, tyrosine, and bityrosine fluorescence.** *A* and *B*, tryptophan fluorescence; *C* and *D*, tyrosine fluorescence; *E* and *F*, bityrosine fluorescence. The results are means of three separate experiments. *Open squares*, fluorescence of PKAI control. *A–D*, *black diamonds*, PKAI saturated with gas (O_2 and sodium formate or N_2O); *black circles*, PKAI irradiated in the presence of the indicated gas.

subunit saturated by the gasses was significantly decreased (50%) and was similarly low in the presence of O_2 and N_2O without protein degradation. This effect may be explained by a modification of the three-dimensional conformation of the free catalytic subunit C due to the bubbling of gasses through the solution. The catalytic subunit was more resistant to gas saturation when part of the holoenzyme than when free in solution. In the holoenzyme, structural interactions between the catalytic and the regulatory subunits might protect the catalytic loop from radical attack.

Effects of Free Oxygen Radicals on cAMP-dependent Protein Kinases—The hydroxyl radical is more reactive and less long lived (in the range of a few tens of nanoseconds) than superoxide. PKAI was more sensitive than PKAII to hydroxyl radicals. The only similar effect of hydroxyl radicals on both enzymes was the significant decrease of phosphotransferase activities either in the presence of cAMP or in the presence of site A- and site B- specific cAMP analogs.

The Sensitivities of PKAI and PKAII to Oxygen Radicals Were Different—The immunoreactivity of both RI and C subunits of PKAI was decreased by exposure to hydroxyl radicals (as assessed by Western blotting), probably due to modification of the epitopes. Moreover, hydroxyl radicals markedly altered N^6-Bnz-cAMP-stimulated PKAI phosphotransferase activity, which suggests that site A was modified. The quenching of tryptophan fluorescence by cAMP, a marker of site A in RI (33), was also decreased. This would explain why both cAMP-binding and cAMP-dependent phosphotransferase activity were altered, because cAMP binding to sites A and B is cooperative: binding to site B favors binding to site A (for a review, see Ref. 18).

In contrast, cAMP binding to RII and the immunoreactivity of RII were not affected by exposure to hydroxyl radicals, although cAMP binding to RI was significantly decreased. The impaired phosphorylation of bovine RIIα by casein kinase II on Ser^{74} or Ser^{76} and by exogenous PKA catalytic subunit (nonirradiated) on Ser^{95} in the N-terminal domain of this regulatory subunit suggests that either the phosphorylation sites or the accessibility to the phosphorylation sites was altered by hydroxyl radicals.

PKAII appeared to be more resistant than PKAI to attack by superoxide free radicals: superoxide radicals did not significantly affect either PKAII cAMP-dependent phosphotransferase activity or the binding of [^{3}H]cAMP or 8-N_3-[^{32}P]cAMP to its RII subunits. Incubation with superoxide radicals significantly altered PKAI. However, the most striking effect was the absence of any PKAI phosphotransferase activity in the presence of N^6-Bnz-cAMP. This cAMP analog binds preferentially to the site A of the RI regulatory subunit. Superoxide radicals also decreased PKAI phosphotransferase activity as determined after stimulation with 8-AHA-cAMP. This cAMP analog binds preferentially to site B of the RI regulatory subunit. In addition, several properties of the RI regulatory subunit ([^{3}H]cAMP binding and 8-N_3-[^{32}P]cAMP binding) were decreased concomitantly with a loss of the tryptophan and tyrosine fluorescence associated with this subunit. Photolabeling of PKAI with 8-N_3-cAMP results in 40% occupancy of cAMP binding site A of RI (Trp^{260}) and 60% of cAMP binding site B (48); superoxide radicals appear to alter the RI subunit at or near both A and B cAMP binding sites. Alternatively, oxidative modifications of other amino acids may alter the allosteric cooperativity between the two cAMP binding sites, and thereby modulate the binding to site B first and subsequently the binding to site A. Furthermore, PKAI is a dimeric protein in which the two monomers are linked by two intrachain disulfide bonds (46). It would therefore be interesting to study the effects of free oxygen radicals on RI dimerization (46).

The free catalytic subunit of these enzymes was altered by the attack of both superoxide and hydroxyl radicals, but more substantially by hydroxyl radicals; the phosphotransferase activity was decreased by 30% (O_2^-) and by 95% ($^{\cdot}OH$).

The subcellular compartmentalization of the different forms of PKA might be of relevance to the modulation of their activities by free radicals. The type II PKA is anchored to membranes and to the cytoskeleton through interaction between its RII regulatory subunit and specific proteins called A kinase anchoring proteins (for a review, see Dell'Acqua and Scott (49)). These A kinase anchoring proteins may also protect the regulatory subunits against the action of free radicals. PKA type I is mainly cytosolic, needs lower concentrations of cAMP to dissociate, and might be more accessible to free radical attack. Different oxygen free radicals have different patterns of diffusion through the cell. The hydroxyl radical is very reactive and alters proteins at its site of production, whereas the superoxide radical O_2^- is more diffusible. In addition, the scavenging systems for free radicals vary between tissues and show specific patterns of subcellular localization (50). Thus, the two types of PKA may be differentially altered by oxidation within a given cell type, depending upon the type of free radicals produced and the types of antioxidant systems present, which are tissue-dependent. Other free radicals such as nitric oxide could also participate in inflammatory diseases such as psoriasis. The uniqueness of the effects on PKA by O_2^- and $^{\cdot}OH$ radicals needs to be studied.

In conclusion, PKAI was more susceptible than PKAII to the gasses N_2O and O_2 used to produce $^{\cdot}OH$ or $O_2^{\cdot-}$, respectively. These gasses probably affect the three-dimensional structure of these enzymes. The C subunit is less sensitive to free radical attack when it is part of the holoenzyme. PKAI is more susceptible to oxygen free radicals than PKAII. Exposure of PKAI and PKAII to hydroxyl radicals causes gross structural modifications due to an alteration to primary structure (at tryptophans and tyrosines). Superoxide radicals have less effect. The exposure of proteins to radiolysis leads to structural perturbations that can greatly influence reactivity with oxygen radicals (for a review, see Ref. 51) and that could explain the different effects observed for PKAI and PKAII. This alteration of PKA by oxygen free radicals may modify signal transduction, inside or outside the cells, which may be involved in inflammatory diseases such as psoriasis.

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