Catecholamines in adipose tissue promote lipolysis via cAMP, whereas insulin stimulates lipogenesis. Here we show that H₂O₂ generated by insulin in rat adipocytes impaired cAMP-mediated amplification cascade of lipolysis. These micromolar concentrations of H₂O₂ added before cAMP suppressed cAMP activation of type IIF cyclic AMP-dependent protein kinase (PKA) holoenzyme, prevented hormone-sensitive lipase translocation from cytosol to storage droplets, and inhibited lipolysis. Similarly, H₂O₂ impaired activation of type II PKA holoenzyme from bovine heart and from that reconstituted with regulatory II subunits. H₂O₂ was ineffective (a) if these PKA holoenzymes were preincubated with cAMP, (b) if added to the catalytic α subunit, which is active independently of cAMP activation, and (c) if the catalytic α subunit was substituted by its C199A mutant in the reconstituted holoenzyme. H₂O₂ inhibition of PKA activation remained after H₂O₂ elimination by gel filtration but was reverted with dithiothreitol or with thioredoxin reductase plus thioredoxin. Electrophoresis of holoenzyme in SDS gels showed separation of catalytic and regulatory subunits after cAMP incubation but a single band after H₂O₂ incubation. These data strongly suggest that H₂O₂ promotes the formation of an intersubunit disulfide bond, impairing cAMP-dependent PKA activation. Phylogenetic analysis showed that Cys-97 is conserved only in type II regulatory subunits and not in type I regulatory subunits; hence, the redox regulation mechanism described is restricted to type II PKA-expressing tissues. In conclusion, phylogenetic analysis results, selective chemical behavior, and the privileged position in holoenzyme lead us to suggest that Cys-97 in regulatory IIα or IIB subunits is the residue forming the disulfide bond with Cys-199 in the PKA catalytic α subunit. A new molecular point for cross-talk among heterologous signal transduction pathways is demonstrated.
PKA Regulation by \( H_2O_2 \)

administration has been reported (7); chronic hyperinsulinenemia resulted in enhanced cAMP production through \( \beta \)-adrenergic receptor activation in adipocytes (8). However, this increase in the cAMP pool should raise lipolysis, but a well known action of insulin is to increase lipogenesis. Such a paradox was explained by demonstrating that continuous high insulin levels impair close apposition of \( \beta \)-adrenergic receptors and PKA; thus, chronic hyperinsulinenemia disrupted the signaling pathway between \( \beta \)-adrenergic receptors and PKA (7). This paper deals with the identification of a novel cross-talk between adrenaline and insulin signaling pathways in isolated adipocytes. Three experimental evidences were considered: (a) insulin stimulates a plasma membrane NADPH oxidase system present in adipocytes and transiently generates \( H_2O_2 \) (9–11); (b) \( H_2O_2 \) mimics insulin action (9, 12, 13); and (c) \( H_2O_2 \) decreased PKA activity in fibroblasts (14) and inhibited PKA activation by dibutyryl cyclic AMP (Bt2cAMP)-activated PKA in isolated adipocytes (15). Therefore, we reasoned that insulin-generated \( H_2O_2 \) might regulate cAMP-dependent PKA activation in adipose cells. There are two major isoforms of cAMP-dependent PKA holoenzymes, type I and type II, both of which exist as tetramers integrated by two catalytic and two regulatory subunits. The regulatory subunit present in PKA holoenzyme, either RI (\( \alpha \) or \( \beta \) subtype) or RII (\( \alpha \) or \( \beta \) subtype), defines the enzyme as type I or type II, respectively. Information on cAMP-dependent protein kinase is abundant, and a summary of the impressive amount of data recorded for this enzyme can be found in a recent review (16) and in the references cited therein.

Oxidative stress can lead to the formation of a large number of protein disulfides (17–19), and although the cytosol is a reducing environment, the thiol status of proteins may be more dynamic than appreciated previously (20). Indeed, it has been recently demonstrated in isolated rat ventricular myocytes that type I PKA is redox-active, forming an interprotein disulfide bond between two cysteines that align antiparallel to each other on different type I regulatory subunits (RIS) in response to cellular \( H_2O_2 \) (21). This oxidative disulfide formation causes subcellular kinase translocation and activation, which phosphorylate their substrate proteins independently of \( \beta \)-adrenergic stimulation and cAMP elevations (21). On other hand, type II PKA holoenzyme, the major isoform present in adipocytes (22), also possess reactive cysteines that can form disulfide bonds between catalytic \( \alpha \) subunits (CoS) and regulatory IIo subunits (RIIoS). Taylor and co-workers (23–26) demonstrated the presence of particularly reactive cysteine residues in PKA holoenzyme, Cys-97 in the RIIoS and Cys-199 in CoS.

The main purpose of our work was to understand the molecular mechanism by which micromolar concentrations of \( H_2O_2 \) might inhibit type II PKA activation by cAMP and to explore the role of disulfide bond formation in this regulation. To undertake this investigation, three type II PKA holoenzymes were used: (a) a cell-free preparation from rat adipocytes containing PKA holoenzyme with C\( \alpha \) and RII\( \beta \) subunits (22); (b) a commercial liophilized enzyme from bovine heart mainly integrated by CoS and RIIoS (27, 28); and (c) a reconstituted holoenzyme prepared with murine C\( \alpha \) and RII\( \alpha \) subunits that were expressed and purified as described previously (29, 30). All three holoenzymes were similarly sensitive to micromolar concentrations of \( H_2O_2 \) in such a way that their cAMP-dependent activation was impaired. As a physiologic consequence of this inhibition in adipose cells, HSL was not translocated from cytosol to fat storage droplets, and lipolysis was slowed. Inactivation by \( H_2O_2 \) was reversed with thiol reagents, and PKA holoenzyme was activated again with cAMP. By use of a fully active \( H_2O_2 \)-insensitive C199A catalytic subunit (26) and supported by phylogenetic analysis of protein kinases, the highlighted Cys residues in PKA holoenzyme that are oxidized by \( H_2O_2 \), preventing cAMP physiologic activation, were identified.

**EXPERIMENTAL PROCEDURES**

Reagents—Kemptide (phosphate acceptor peptide; Leu-Arg-Ala-Ser-Leu-Gly), Bt2cAMP, HEPES, MES, MOPS, dithiothreitol (DTT), GSH, cAMP, GTP\( \gamma \)S, insulin, Epac agonist (8-pCPT-2′-O-Me-cAMP), PKA inhibitor H89, thioeductin, thioeductin reductase, collagenase type II, catalase, crude lyophilized PKA from bovine heart, and lyophilized PKA catalytic subunit from bovine heart were obtained from Sigma. Proteinase cocktail and auranofin were obtained from MP (Fountain Parkway, Solon, OH). The Amplex Red kit was obtained from Molecular Probes, Inc. (Eugene, OR). \( H_2O_2 \) was obtained from Merck. Phosphocellulose filter paper P-81 and GF/C filters were purchased from Whatman, Inc. (Florham Park, NJ), whereas Sephadex G-50 was obtained from Ame- sham Biosciences. \([\gamma-32P]ATP \) was obtained from PerkinElmer Life Sciences, and part was a generous gift from M. Tuena de Gómez-Puyou at Institute of Cellular Physiology, National Autonomous University of Mexico with specific activity of \( 1.5 \times 10^5 \) cpm/nmol \([\gamma-32P]cAMP \) was purchased from Amer- sham Biosciences, and synthetic peptide GPRLELRPRPQQSQRS was prepared by Biosynthesis, Inc. (Lewisville, TX). Rabbit antiserum was prepared by C. Agundis and M. A. Pereira from the Department of Biochemistry, Faculty of Medicine, National Autonomous University of Mexico. The antiserum was raised against the synthetic peptide just mentioned, derived from rat HSL (amino acid sequence 326–341) (31), and bound to fibrinogen. Specificity of the antibody was evaluated by an ELISA inhibition assay and by direct ELISA with the peptide alone (32). Purified murine PKA type II proteins (wild type catalytic \( \alpha \) subunit, its C199A mutant, and regulatory RII\( \alpha \) subunits) were prepared in the laboratory of Dr. Susan S. Taylor (University of California, San Diego) (29, 30), and they were a generous gift from her.

Animals—Male Wistar rats weighing 200–240 g fed ad libitum with a commercial diet (Purina, México) and free access to water were used. All experiments were conducted in accordance with the Federal Regulations for Animal Care and Use (NOM-062-ZOO-1999, Ministry of Agriculture, Mexico) and were approved by the Ethics Committee of the Faculty of Med-icine, National Autonomous University of Mexico.

**Adipocyte Isolation and Measurement of Lipolysis**—Animals were fasted for 16 h and decapitated. Subsequently, epidydial fat pads from two rats were immediately removed to isolate adipocytes following recommendations to obtain cells with low cAMP basal levels (33). In brief, Ringer-Krebs buffer was enriched with 25 mM HEPES, 2.5 mM Ca\( Cl_2 \), 2 mM glucose, 200
PKA Regulation by H$_2$O$_2$

nm adenosine, and bovine serum albumin (bovine serum albumin fraction V, fatty acid-free) either at 1 or 4%, as detailed later; pH was adjusted at 7.4. One g of minced fat pad from two rats was digested in 10 ml of collagenase (1 mg/ml) for 30 min, with vigorous shaking, in the Ringer-Krebs-enriched buffer supplemented with 1% bovine serum albumin. Cells were filtered through nylon cloth and washed three times by centrifugation (1 min each) at 220 × g. Wet packed adipocytes were weighed to report glycerol release by wet weight as an index of lipolysis, which was assayed using 100 μl of packed adipocytes, incubated during 30 min at 37 °C in a total volume of 1 ml of 4% bovine serum albumin in the Ringer-Krebs-enriched buffer in which Bt$_2$cAMP, H$_2$O$_2$, catalase, insulin, 8-pCPT-2'-O-McAMP, or H$_8$9 were dissolved appropriately to reach the final concentrations indicated in the figures. Adipocytes were dispersed during incubation by shaking at 160 cycles/min. Lipolysis, which Bt$_2$cAMP, H$_2$O$_2$, catalase, insulin, 8-pCPT-2'-O-McAMP, or H$_8$9 were dissolved appropriately to reach the final concentrations indicated in the figures. Adipocytes were dispersed during incubation by shaking at 160 cycles/min. Lipolysis was stopped by transferring tubes from 37 °C to an ice bath for 5 min. Tubes were immediately centrifuged at 10,000 × g at 4 °C for 10 min. A 300-μl aliquot from the solution lying below the fat cake was used to measure glycerol (34).

NADPH-dependent H$_2$O$_2$ Generation System Activity—The procedure described to measure NADPH oxidase system activity in human adipocytes was followed (35). In brief, 100 μl of packed rat adipocytes obtained as described previously were suspended in 900 μl of an ice-cold lysing medium containing 20 mM MES pH 8.5, 2 mM MgCl$_2$, 1 mM CaCl$_2$, 5 mM KCl, and 100 μM of mixture protease inhibitor. Cells were lysed after vigorous mixing for 5 min with the aid of a vortex. Lysed cells were spun at 1,000 × g for 20 min at 4 °C, supernatant was discarded, and the plasma membrane-containing precipitate was suspended in the activation buffer containing 30 mM MOPS, pH 7.5, 120 mM NaCl, 1.4 mM CaCl$_2$, 5 mM MgCl$_2$, and 10 mM NaHCO$_3$. Centrifugation was repeated under identical conditions, supernatant was discarded, and the precipitate was suspended in the activation buffer supplemented or not with MnCl$_2$, GTPγS, cAMP, or insulin, as detailed in the figure legends. The NADPH oxidase system present in plasma membranes was activated by incubation at 25 °C during 25 min. Then, samples were centrifuged under the same conditions, and the precipitate was suspended and washed twice in catalysis buffer containing 30 mM MES, pH 5.8, 120 mM NaCl, 4 mM MgCl$_2$, 1.2 mM KH$_2$PO$_4$, 1 mM NaN$_3$, and 10 mM FAD. Samples were spun and suspended in the same buffer, and the catalytic reaction was started with 250 μM NADPH and incubated for 30 min at 37 °C. Reaction was stopped, transferring tubes from 37 °C to an ice bath for 5 min to measure H$_2$O$_2$ (36). A 5-μl aliquot from mix reaction was used with 45 μl of 50 mM sodium phosphate buffer, pH 7.4, and 50 μl of Amplplex Red solution (commercial kit); samples were maintained during 30 min at room temperature in the dark, and absorbance was measured at 560 nm. Amplplex Red reagent reacts with H$_2$O$_2$ in a 1:1 stoichiometry to produce resorufin, the red fluorescent oxidation product that has an extinction coefficient of 54,000 cm$^{-1}$ M$^{-1}$ at 560 nm (36).

Assay of PKA Activity—Once rat adipocytes were prepared as described, a cell-free preparation suitable for assaying PKA enzyme activity was obtained (15). In short, 100 μl of isolated adipocytes was mixed with 900 μl of Ringer-Krebs buffer supplemented with 5 mM glucose and 2% bovine serum albumin and incubated during 1 h at 37 °C. Then the mixture was centrifuged at 2,400 × g during 5 min. The supernatant was eliminated, and the sediment containing adipocytes was disrupted in 350 μl of lysis buffer (50 mM Tris-HCl, pH 7.8, 0.33 mM sucrose, 1 mM MgCl$_2$, and protease inhibitors), and after vigorous shaking the sample was centrifuged at 2,400 × g for 4 min to obtain a floating fat cake, an intermediate layer, and the sediment. The intermediate layer was the source for measuring PKA activity in adipocytes. To measure PKA catalytic activity, aliquots of this cytosolic extract, either passed or not through Sephadex columns (see below), were mixed with 30 μl of 50 mM MOPS buffer, pH 7.0, containing 250 μg/ml bovine serum albumin, 100 μM ATP, 10 mM MgCl$_2$, and [γ$^{32}$P]ATP (specific activity of 0.5 × 10$^6$ cpm/μmol) final concentration in incubation mixture. To analyze the effect of H$_2$O$_2$ on cAMP-mediated PKA activation, hydrogen peroxide was added to the enzyme either before, simultaneously with, or after the cyclic nucleotide. The catalytic reaction was started with 80 μM Kemptide in a total volume of 60 μl. At the end of the incubation period, 25 μl of reaction mixture was placed on a piece (1.5 × 1.5 cm) of Whatman P-81 filter paper, washed three times with 5 ml of 5% phosphoric acid, and placed in a vial with scintillation liquid to measure radioactivity.

Gel Filtration to Separate Small Molecules from PKA—A 100-μl PKA aliquot from adipocyte lysates was preincubated with H$_2$O$_2$, and their respective controls were preincubated without H$_2$O$_2$. Then they were carefully loaded into an insulin syringe containing 700 μl of previously equilibrated Sephadex G-50 and centrifuged at 3,000 rpm for 3 min at 4 °C (37). The effluent containing the macromolecules separated from the small molecules was collected in 1.5 ml of Eppendorf tubes and utilized to assay PKA enzymatic activity as described above. In some experiments as detailed in the figure legends, the enzymatic reaction was measured in the presence of GSH or DTT.

Translocation of HSL from Cytosol to Fat Storage Droplets—To define the HSL subcellular mobilization, both the floating fat cake containing fat storage droplets and the intermediate layer of adipocyte lysates obtained as described under "Assay of PKA Activity" were mixed and incubated in buffer with either 1 mM cAMP or 1 μM H$_2$O$_2$. Western blot analysis was conducted in the former two fractions (38). Proteins resolved in SDS-PAGE were transferred to nitrocellulose membranes and exposed to rabbit anti-HSL antiserum. Visualization was performed with a lumino kit (Super Signal West Pico kit; Pierce), and band quantitation was effected by densitometry using a Fotodyne apparatus (Fotodyne, Inc., Hartford, WI).

Assay of Thioredoxin Reductase Activity—Two type of assays were conducted with thioredoxin reductase activity: one to revert the formation of an interprotein S–S bond and the other to verify its functional presence in adipocytes by measuring the enzyme’s catalytic activity. An aliquot of lysated intermediate layer adipocytes was mixed in 0.1 ml Tris-HCl buffer, pH 7.8, supplemented with 0.1 mM EDTA, 1.4 mM 5,5'-dithiobis(nitrobenzoic acid), and 310 μM NADPH. The difference of 5,5' dithiobis(nitrobenzoic acid) oxidation in the presence and absence of auranofin, a specific inhibitor of thioredoxin reductase enzyme (39), was registered as the enzyme activity value. To hinder formation of an S–S bond by the thioredoxin reduc-
PKA Regulation by H$_2$O$_2$

**FIGURE 1. Lipolysis as adipocyte response to insulin, cAMP analogs, and H$_2$O$_2$.**

- a. glycerol release by isolated rat adipocytes incubated for 1 h with the additions indicated in the figure: 10 $\mu$M Bt2cAMP; increasing concentrations of insulin (from left to right, 10$^{-10}$, 10$^{-9}$, and 10$^{-8}$ M); and 1,000 units of catalase. Shown are comparisons of insulin versus insulin plus catalase ($n = 3$). b. generation of H$_2$O$_2$ by NADPH oxidase from cell plasma membrane of isolated adipocytes with the addition indicated in the figure: 1 mM insulin, 3 mM Mn$^{2+}$, 10 $\mu$M Bt2cAMP, and 10 $\mu$M Bt2cAMP, $n = 3$. c. concentration-response curve for the effect of H$_2$O$_2$ on Bt2cAMP-stimulated glycerol release in isolated rat adipocytes. Basal glycerol release in the absence of cAMP (●), in the presence of 10 $\mu$M Bt2cAMP (○), or in the presence of 1 mM Bt2cAMP (□). Shown are comparisons of control versus Bt2cAMP and Bt2cAMP without H$_2$O$_2$ versus Bt2cAMP plus H$_2$O$_2$, $n = 5$. Inset, initial velocity ($V_0$) with H$_2$O$_2$/initial velocity without H$_2$O$_2$ ($V_0$) versus the logarithm of [H$_2$O$_2$] was plotted to calculate IC$_{50}$ value for H$_2$O$_2$. d. glycerol release by isolated rat adipocytes incubated for 1 h with the additions indicated in the figure: 10$^{-3}$ M Bt2cAMP, 10 $\mu$M 8-pCPT-2'-O-Me-cAMP, and 25 $\mu$M H89 ($n = 3$).

**Competition Binding Experiments between cAMP and H$_2$O$_2$**

- The adipocyte extract intermediate layer was used as a PKA source for competition binding experiments (36). Two hundred $\mu$g of protein were incubated for 15 min with 14 different concentrations of H$_2$O$_2$ (3 $\times$ 10$^{-10}$ to 3 $\times$ 10$^{-3}$ M), and then 0.4 $\mu$M [$H$]cAMP (59 Ci/mmol) was added, and incubation continued for 1 h at 25°C as described (40). Thereafter, the reaction mixture was filtered through Whatman GF/C filters using a Brandel cell harvester (Biomedical Research and Development Laboratories, Gaithersburg, MD); liquid scintillation was added, and the sample was counted in a liquid scintillation spectrometer at 60% efficiency (LS 6000; Beckman, Fullerton, CA). Data were analyzed by nonlinear regression with the EBDA-ligand program (Biosoft, Ferguson, MO).

**Acid Gels and Western Blot Analysis**

- SDS-polyacrylamide gels at pH 2.4 were prepared as described by Dame and Scarborough (41), using 10 and 5% acrylamide for resolving and stacking gels, respectively. A 60-µl aliquot of the adipocyte extract free of cells and lipids was incubated at 37°C in the presence of cAMP and H$_2$O$_2$ for the times indicated in the figure legend. At the end of the incubation time, samples were boiled for 5 min and then loaded onto the gel (30 µg/lane). Electrophoresis was run at room temperature (20–22°C) during 4 h at 75 mA. After electrophoresis, proteins were transferred to a nitrocellulose membrane. Western blot analysis was performed using polyclonal antibodies against PKA catalytic α subunit (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). After the membrane was thoroughly washed, it was incubated with appropriate peroxidase-conjugated secondary antibody at 4°C (Jackson, CA). Protein bands were visualized by using a luminol/enhancer system (Super-Signal West Pico chemiluminescence kit; Pierce). A detailed description of the above mentioned procedure is given in Ref. 32.

**Phylogenetic Analysis**

- The search for protein kinase sequences was performed at the National Center for Biotechnology Information World Wide Web site (43). The integrated data base retrieval system ENTREZ (44) was used to access the NCBI data base. The search for homologous sequences was performed with gapped BLASTP utilizing default penalties and BLOSUM62 substitution matrix (45). Human PKA sequences comprised the starting point in the search for homologous sequences.

Progressive multiple-sequence alignment was calculated with the ClustalX package (46), employing secondary structure-based penalties. Alignment was manually corrected according to the results of gapped BLAST, and three-dimensional alignments were obtained from the ENTREZ three-dimensional structure data base (47). Phylogenetic analyses were performed with MEGA 3.1 software (48) using maximum parsimony, minimum evolution, neighbor joining, and unweighted pair group method using arithmetic averages (UPGMA) methods with the aid of the empirical Jones–Taylor–Thornton amino acid substitution model. Differences among amino acid sequences were corrected for multiple substitutions, assuming γ distribution for rate variations among sites. The $γ$-shaped parameter ($α = 1.0$) was estimated with the Whelan-Goldman matrix of substitutions and the eight-category discrete $γ$ model,
**RESULTS**

**Lipolysis Inhibition with H$_2$O$_2$—**

The well known stimulation of glycerol release in isolated adipocytes by Bt$_2$cAMP was progressively inhibited by growing doses of insulin (Fig. 1d). It is noteworthy that this inhibitory action of insulin was observed at hormone concentrations up to 4 orders of magnitude lower than those of Bt$_2$cAMP. To verify that insulin-generated H$_2$O$_2$ was the signal responsible for this observed inhibition, catalase was added to degrade H$_2$O$_2$. Catalase, an enzyme highly specific for H$_2$O$_2$, was preferred to dipheno-leneiodonium, a nonspecific NADPH oxidase system inhibitor used by other authors with a similar objective: to block production of cellular H$_2$O$_2$ (11). As shown in Fig. 1a, catalase abolished the inhibitory action of insulin on Bt$_2$cAMP-stimulated glycerol release. Given the paramount importance of H$_2$O$_2$ in these experiments, direct evidence of insulin-generated H$_2$O$_2$ in adipocyte plasma membranes prepared from rat epididimus was explored. As expected, insulin in combination with Mn$^{2+}$ or GTP$_\gamma$S promoted H$_2$O$_2$ production from NADPH, (Fig. 1b), similar to the manner in which this occurs in plasma membranes of human adipocytes incubated with GTP$_\gamma$S, NADPH, and insulin (31) and in 3T3-L1 adipocytes challenged with insulin (11, 50). In agreement with the already presented results, experiments in Fig. 1c demonstrate that H$_2$O$_2$ externally added to isolated rat adipocytes inhibited Bt$_2$cAMP-activated glycerol release, with an IC$_{50}$ value of $\sim 5 \times 10^{-5}$ M, a concentration 20 times lower than that reported previously ($10^{-3}$ M) used to inhibit Bt$_2$cAMP-activated PKA in isolated adipocytes (16). Experiments to analyze the role of Epac in adipocyte cell lipolysis response were conducted, because previous reports showed the participation of Epac in two cases of cross-talk between insulin and cAMP signaling pathways (6, 7). As shown in Fig. 1d, the PKA inhibitor H89 (51) prevented release of Bt$_2$cAMP-promoted glycerol in adipocytes, and the Epac analog, 8-pCPT-2'-O-Me-cAMP (52) did not induce a lipolytic response. Hence, cAMP action on PKA is the only route that activates glycerol release in adipocytes, and H$_2$O$_2$, either added or generated in adipocyte plasma membranes by insulin, is involved in inhibition of cAMP-activated glycerol release in these cells.

**PKA Regulation by H$_2$O$_2$**

Utilizing TREE-PUZZLE 5.2 (49). Branch point confidence limits were estimated by 500 bootstrap replications. The complete names of organisms included in the phylogenetic analysis are provided in supplemental Appendix 1.
**PKA Regulation by H₂O₂**

H₂O₂ Inhibits cAMP-mediated Activation of PKA from Adipocytes—The initial step in cAMP amplification cascade is PKA activation by the cyclic nucleotide. In consequence, PKA catalytic activity present in cell-free lysates of isolated adipocytes was challenged with H₂O₂ to identify the target molecule responsible for lipolysis inhibition. A dose-response inhibition of H₂O₂ on PKA activity was observed in holoenzyme samples using three different cAMP concentrations measured in the presence or absence of the catalase inhibitor 4-aminotriazole (53) (Fig. 2a). cAMP-mediated activation of PKA was modulated with lower doses of H₂O₂; a statistically significant inhibition was obtained with 10⁻³ M H₂O₂ despite the presence of 10⁻⁵ M cAMP (Fig. 2a). This suggests independent binding sites for the action of each of these modulators. To substantiate this point and to gain insight into the mechanism by which H₂O₂ prevents PKA activation or enzymatic activity, binding experiments employing [³H]cAMP as the adipocyte enzyme ligand were performed. Results showed no displacement of the cyclic nucleotide by H₂O₂, confirming different binding sites for H₂O₂ and cAMP on PKA holoenzyme (results not shown). Furthermore, the inhibitory action of H₂O₂ was restricted to PKA holoenzyme activation, in that H₂O₂ was unable to inhibit the enzymatic activity of the already cAMP-activated holoenzyme (i.e. of the catalytic subunit); however, if the holoenzyme was first preincubated with H₂O₂, its activation by cAMP was prevented, and enzyme activity was completely inhibited (Fig. 2b).

**FIGURE 4. Effect of H₂O₂-mediated PKA inactivation on HSL mobilization.** To study subcellular HSL mobilization, 50 μl of the floating fat storage droplets and 50 μl of the intermediate layer of adipocytes containing PKA were mixed and incubated with 100 μM ATP. In addition, 1 mM cAMP and 1 μM H₂O₂ were included as follows. C (control), incubated for 25 min in the absence of cAMP and H₂O₂; cAMP, incubated first with cAMP during 15 min and then with H₂O₂ for 10 additional min. H₂O₂, incubated for 15 min with H₂O₂, and then with cAMP for 10 additional min. To assess HSL localization at the end of incubation periods, tubes were vortexed vigorously and centrifuged at 13,000 x g at 4 °C for 15 min to obtain a lower layer (corresponding to cytosol) and the floating fat cake (corresponding to lipid droplets). The cytosolic fraction was aspirated from below the solidified fat cake, and 100 μl of cytosol was mixed with an equal volume of sample buffer for SDS-PAGE. The fat cake fraction was respun at 13,000 x g at 4 °C for 15 min, and any contaminating cytosol was aspirated and discarded. The fat cake was warmed to room temperature, 100 μl of SDS sample buffer was added, and the solution was vortexed thoroughly. Following centrifugation at 13,000 x g at 4 °C for 15 min, the fat cake protein extract was aspirated from below the floating fat layer for PAGE. These samples were applied to 10% SDS-polyacrylamide gels. Following electrophoresis, proteins were transferred to nitrocellulose membranes, and HSL was identified with a polyclonal antibody. The experiment shown is representative of five independent experiments.

**FIGURE 5. Thiol reagents reversed H₂O₂ action on inactivated PKA.** a, PKA reactivation with GSH. The enzyme was preincubated for 10 min at 37 °C with 1 μM H₂O₂ or without H₂O₂ and then passed or not through Sephade columns. The black bars indicate enzyme activity when samples were not passed through Sephade columns, and gray bars denote samples passed through Sephade columns previously equilibrated with 50 mM Tris-HCl buffer, pH 7.8, supplemented with 1 mM MgCl₂. Eluted macromolecules or samples not passed through the columns were incubated to measure PKA activity as detailed under “Experimental Procedures” except for the reagents indicated in the figure: 100 μM cAMP and 9 mM GSH. b, PKA reactivation with dithiothreitol (DTT). The enzyme was incubated under similar conditions as described in a, but GSH was substituted by 1 mM DTT. c, PKA reactivation with thioredoxin plus thioredoxin reductase. PKA enzyme source was incubated for at least 10 min with the following reagents: 1 mM cAMP and 10 μM H₂O₂ added simultaneously, plus 0.4 units of thioredoxin reductase, 18 μM thioredoxin, and 80 μM NADPH. In this experiment, samples were not passed through Sephade columns. Incubations in the presence of thioredoxin plus thioredoxin reductase were 10, 20, and 30 min for the last three bars from left to right; n = 3 for the experiment in each panel.
PKA Regulation by H$_2$O$_2$

H$_2$O$_2$-mediated Oxidation of PKA Is Reversible—A requisite to consider H$_2$O$_2$-mediated oxidation and inactivation of PKA as a leading and physiologic step in homeostasis is that it should be easily impaired or reversed in order to respond to alternating cellular conditions. Elimination of H$_2$O$_2$ alone by molecular filtration through small Sephadex columns was not sufficient to reactivate PKA (Fig. 5, a and b). Nonetheless, the addition of thiols retrieved PKA reactivation by cAMP, with dithiothreitol functioning better than glutathione (Fig. 5, a and b). Seeking a physiologic antioxidant to substitute for dithiothreitol, the thioredoxin- and thioredoxin reductase-integrated system was assayed, based on the fact that in mammalian cells it modulates the activity of a number of proteins through thiol/disulfide reactions (56). PKA catalytic activity was restored after longer incubation periods with thioredoxin reductase system (Fig. 5c), indicating a higher reactivation of the enzyme toward cAMP due to progressive disappearance of interchain disulfide bonds, generated early by H$_2$O$_2$ oxidation. Therefore, an extra mechanism of PKA regulation is advanced that reverts cAMP-insensitive PKA into cAMP-sensitive PKA. Additionally, thioredoxin reductase expression in epididymal rat adipocytes was demonstrated by measuring enzyme activity. A value of 0.68 ± 0.16 μmol/mg of protein/min at 25 °C ($n = 3$) was recorded.

H$_2$O$_2$ Forms an Interchain Disulfide Bond between PKA Catalytic and Regulatory Subunits—All data presented point toward the oxidation of -SH groups in PKA holoenzyme by means of low H$_2$O$_2$ doses, producing a disulfide bond that impaired activation of the enzyme by cAMP. Based on previous reports on the particular reactivity of different type II PKA-

pared from lysated adipocytes. In control experiments, when this system was incubated in the absence of PKA and H$_2$O$_2$, HSL was recovered in the cytosol (Fig. 4). When the system was incubated with cAMP prior to being incubated with H$_2$O$_2$, HSL was translocated from cytosol to fat storage droplets (Fig. 4), as described previously by the Londos group (38), but if PKA was incubated with H$_2$O$_2$ before the addition of cAMP, H$_2$O$_2$ prevented HSL translocation from cytosol to fat storage droplets, despite the late presence of cAMP (Fig. 4). Because each tube initially contained the same amount of HSL, the results obtained are due to a different distribution of HSL between the cytosol and fat cake fractions; thus, HSL in one fraction is the control of the other. Of physiologic relevance is the fact that H$_2$O$_2$, by blocking cAMP activation of PKA, impaired PKA-mediated phosphorylation of HSL and the translocation of HSL from cytosol to fat storage droplets (Fig. 4), resulting in a decrease in the glycerol release rate (Fig. 1, a and c).
holoenzyme cysteines (23–26), we presumed that the holoenzyme in the presence of H₂O₂ promotes formation of an inter-subunit disulfide bond between Cys-97 in RIαS and Cys-199 in CaS, thus abolishing cAMP-mediated release of regulatory and catalytic subunits and therefore maintaining CaS inactive. In contrast, initial incubation with cAMP will favor holoenzyme dissociation and activation, and subsequent addition of H₂O₂ cannot form the disulfide bond and prevent its activation. A straightforward test of this hypothesis can be achieved by using wild-type and mutant proteins. The CaS mutant with Cys-199 substitution by Ala (C199A, conserving full catalytic activity) might be confirmatory; the holoenzyme formed between C199A and wild-type regulatory subunit should not be susceptible to inhibition by H₂O₂ due to the impossibility of forming the corresponding interchain disulfide bond. Experiments with pure PKA subunits (25, 29, 30) fully confirmed our presumptions. Two types of holoenzymes were reconstituted by dialysis (23), one utilizing wild-type CaS and wild-type RIαS, here identified as wild type, and the other employing C199A subunit and the same wild-type RIαS, herein identified as C199A. Both holoenzymes were challenged with H₂O₂ prior to activation by cAMP. Cyclic AMP-activation was prevented by H₂O₂ in the wild-type CaS-containing holoenzyme, whereas the holoenzyme with C199A mutant subunit was H₂O₂-insensitive (Fig. 6a). The following controls were recorded. Both holoenzymes were activated by cAMP; preincubation with cAMP suppressed negative regulation by H₂O₂; and catalytic wild-type and C199A subunit activity were not inhibited by H₂O₂ (Fig. 6a).

To confirm the formation of a disulfide bond in PKA holoenzyme, native electrophoresis of the untreated adipocyte extract was carried out. As shown in Fig. 6b (lane 1), a single band on the gel recognized by the anti-CaS antibody and corresponding to the PKA holoenzyme was obtained. Preincubation of the extract with cAMP resulted in the presence of the same band (Fig. 6c, lane 2), due to cAMP-mediated dissociation of CaS from the regulatory subunit. Surprisingly, the anti-CaS antibody recognized no band on the gel when the extract was preincubated with H₂O₂ prior to the addition of cAMP (Fig. 6c, lane 3). In contrast, the 38-kDa band was observed in all cases if samples were treated with DTT (Fig. 6c, lane 6). We have no explanation for the undetected heterodimer band at the expected position.

Phylogenetic Analysis of Catalytic and Regulatory cAMP-dependent PKA Subunits—An analysis of this type could be useful to predict, based on the amino acid sequences, the cells and organisms in which the regulatory mechanism described herein can or cannot operate. This analysis aided in understanding the mechanism of regulation, the metabolic consequences, and the foreseen generalization to other kinases. Results showed that Cys-199 in catalytic subunits is strictly conserved in all reported cAMP-dependent protein kinases and even in other related protein kinases (see supplemental Table 1) (Fig. 7). In contrast, Cys-97 in regulatory subunits is conserved solely in RIα and -β ortholog proteins; RIα, RIβ, and other nonvertebrate homologous protein sequences possess Ser at this position (Fig. 8).

PKA catalytic subunits comprise the catalytic cAMP-dependent protein kinase subfamily present in protista, fungi, and animals but absent in plants (Fig. 7). They are closely related with the group of protein kinase x that comprise type I cAMP-dependent protein kinases (58). Protein kinases x are present in both vertebrates and invertebrates. The catalytic cAMP-dependent protein kinase subfamily possesses different isoforms originated by several independent duplications in each taxon they are present: protista, fungi, and animals. The PKA catalytic subunit phylogenetic tree (Fig. 7) demonstrates that all protein sequences from vertebrates group together, suggesting that the origin of their different catalytic subunits occurred in parallel to vertebrate evolution. Thus, amphibians possess two different PKA catalytic subunits (CaS and CβS), mammals possess three (CaS, Cβ1S, and Cβ2S), and primates, including humans, possess four (CaS, Cβ1S, Cβ2S, and CyS). Interestingly, independent duplication events have taken place recently in fish. In this way, the zebrafish (Danio rerio) and the pufferfish (Tetraodon nigroviridis) each has three PKA catalytic subunits (one CaS plus two CβS, and two CaS plus one CβS, respectively). Several gene duplications, independent of those observed in vertebrates, have also occurred. Thus, although several invertebrates possess only a single PKA catalytic subunit, others possess two (Anopheles gambiae, Apis mellifera, and Schistosoma japonicum) or even three isoforms (Aplysia californica).

FIGURE 7. Phylogenetic tree constructed with the maximum parsimony method that includes closest relatives to the CaS subunit. Similar topologies were obtained using neighbor joining, minimum evolution, and unweighted pair group methods with arithmetic mean (UPGMA) algorithms. Trees were calculated using MEGA 3.1 software (48). The vertical bars indicate the symbol approved by the Human Genome Nomenclature Committee (labels in capital letters) and/or the taxonomic group (labels in italic type) to which the protein sequence belongs. For nonmammalian protein sequences, names were assigned in agreement with Human Genome Nomenclature Committee names. Sequence names are indicated according to a SwissProt-like identifier (gene_organism), followed by the data base accession number and protein amino acid length. The sequences shown correspond to subdomain VIII (69) of CaS (residues 194–212), including the conserved Cys-199 (highlighted in yellow); numbering is according to rat CaS protein sequence (highlighted in orange); additional conserved residues are also highlighted. The numbers on branches are support values from 500 bootstrap replicates. The phylogenetic trees were rooted using the PknB serine/threonine kinase from Mycobacterium tuberculosis (42). The complete organism name included in phylogenetic analysis is provided in supplemental Appendix 1. PRKAC, catalytic cAMP-dependent protein kinase.
PKA Regulation by H₂O₂

On the other hand, PKA regulatory subunits comprise the regulatory cAMP-dependent protein kinase subfamily present also in protista, fungi, and animals but absent in plants (Fig. 8). They are closely related with the regulatory domain of the cGMP-dependent protein kinase subfamily. The catalytic cAMP-dependent protein kinase subfamily possesses different isoforms originated by several independent duplications in each taxa in which they are present: protista, fungi, and animals. In invertebrates, there exist two different regulatory cAMP-dependent protein kinase proteins (RIS and RIIS), and in vertebrates there are four different regulatory cAMP-dependent protein kinase proteins (RαS, RβS, RIIαS, and RIIβS), although some recent additional duplications have taken place in specific taxa. For example, the African clawed frog (Xenopus laevis) and the zebrfish (D. rerio) possess two RαS proteins.

**DISCUSSION**

This paper deals with identification, at the molecular level, of a cross-talk between the signaling of the two most important hormones in regulating lipid storage metabolism: insulin and adrenaline. This finding might be a key to better understanding of the dynamic control of adipose tissue, the most abundant reserve of energetic molecules in mammals. Type II PKA becomes the central sensor responding to adrenal signal by activation or to insulin signal by blunting lipid degradation. The relevance of the physiologic role of cAMP in adipocyte adrenaline signaling (Fig. 1d) is so general that indeed it is included in biochemistry and physiology textbooks. In regard to insulin signaling in adipocytes, one part of the elicited response by this hormone is the generation of H₂O₂ (9–11), although the physiologic role of the latter has been incompletely studied (50). H₂O₂ generated in isolated epididymal rat adipocyte cell membranes by insulin (Fig. 1b) was required, at least in part, to produce insulin-promoted inhibition of β₃CAMP-stimulated glycerol release, because removal of H₂O₂ by added catalase prevented lipolysis inhibition by insulin (Fig. 1a). Based on NADPH oxidase localization in plasma membrane of adipocytes (10, 11) (Fig. 1b) and taking into consideration its function, activation of this enzyme by insulin will raise the H₂O₂ pool in adipocyte extracellular space, resulting in H₂O₂ diffusion into the cell. The addition of catalase will degrade H₂O₂ in the extracellular space. In the complex response of adipocytes to insulin (11, 50), the presence of H₂O₂ alone was sufficient to inhibit stimulated lipolysis (Fig. 1c); thus, it allowed us to calculate an IC₅₀ value for H₂O₂ within the range of 5 × 10⁻⁵ M (Fig. 1c, inset). In addition, it is worth noting that 10⁻⁷ M H₂O₂ significantly decreased stimulated glycerol release by 10⁻⁴ M β₃CAMP (i.e. at doses of H₂O₂ that are 3 orders of magnitude lower than β₃CAMP doses).

The next step in this study was to analyze the effect of H₂O₂ on PKA adipose cell activity. When cAMP and H₂O₂ were added simultaneously (Fig. 2a), statistically significant inhibition was recorded with H₂O₂ concentrations as low as 10⁻⁶ M and with cAMP concentrations 3 or 4 orders of magnitude higher than those of H₂O₂; as expected, this effect was enhanced if catalase was inhibited with aminotriazol (Fig. 2a). Preincubation of the extract from adipocytes with H₂O₂ oxidized type IIB PKA holoenzyme and prevented its activation upon cAMP addition (Fig. 2b). Consequently, H₂O₂ impeded HSL translocation from cytosol, where it is localized under basal conditions, to fat storage droplets, where it is translocated after phosphorylation by activated PKA (4). This information indicates that in the whole cell, a molecular point for controlling cAMP-activated lipolysis comprises PKA activation.

Results obtained with type II PKA from bovine heart allowed us to generalize the finding, at least at the molecular level. PKA type II holoenzyme, either α subtype from bovine heart or β subtype from adipocytes, has the same response to H₂O₂. Nevertheless, generalization of action at the cellular level to regulate metabolic routes, such as lipolysis, requires further experimentation. Other cell types might require characteristics analogous to those appearing to favor the reaction of H₂O₂ with PKA: extracellular generation of H₂O₂ not being accessible to cellular catalase; specialized aquaporins to translocate H₂O₂ throughout the membranes, as reported for some mammalian cells (59); and some protein kinase A-anchoring protein targeted to plasma membrane with the ability to form complexes with type II PKA (60).

The results obtained with purified PKA subunits were decisive in confirming the formation of an intersubunit disulfide bond after mild holoenzyme oxidation with low H₂O₂ concentrations. RIIαS formed this interchain bond only with wild-type Cos and not with C199A mutant (Fig. 6a); thus, Cys-199 is absolutely required to generate the S–S bond that suppressed PKA holoenzyme activation. Identification of Cys-97 in RIIαS as the amino acid residue that participates in intersubunit disulfide bonding in the oxidized heterodimer was strongly suspected after demonstration of its particular chemical behavior. Cys-97 in RIIαS and Cys-199 in Cos are reactive cysteine residues selectively protected from alkylation in the holoenzyme, but these are alkylated only when the subunits are dissociated (23), indicating that in the holoenzyme, these residues without forming an S–S bond are mutually protected and bur-
ied in the interface between RIIα and Cα (23). In addition, cross-linked interchain disulfide bonding has been reported between RII and C subunits generated by disulfide interchange using either RII or C subunit modified with 5,5'-dithiobis(2-nitrobenzoic acid) or 4-(azidophenylthio)phthalimide (25). In addition, interchain disulfide bonds also form spontaneously when RII and C subunits are dialyzed at pH 8.0 in the absence of reducing agents (25). In the latter three cases, the specific amino acid residues that participate in intersubunit disulfide bonding have been identified as Cys-97 in the RII subunit and Cys-199 in the C subunit (25). Phylogenetic analysis shows that Cys-97 is highly conserved solely in all type II PKA regulatory subunits studied to date (Fig. 8), and this position is substituted by Ser in type I PKA regulatory subunits. In this manner, phylogenetic analysis indicates that Cys-97 is a conserved residue for guaranteeing the full physiologic role of type II PKA. In conclusion, and based on all previously summarized information, the interchain disulfide bond whose generation is favored by H2O2 must be that which is formed between Cys-97 in the RII subunit and Cys-199 in the C subunit (25).

Based on these findings, we propose that the PKA enzyme in adipocytes can be found in an active form after its activation with cAMP and in two inactive forms: a tetrameric holoenzyme susceptible to being activated immediately by cAMP or inactivated by H2O2, forming an oxidized tetrameric holoenzyme containing an S–S bond between regulatory and catalytic subunits that cannot be activated by cAMP. The presence of cytosolic tetrameric PKA requiring preactivation prior to regular cAMP activation (Fig. 5) advanced the existence of a system to convert this into the “preactive” holoenzyme. In our experiments, this was achieved by thioredoxin plus thioredoxin reductase (Fig. 5c), but other systems with the following characteristics might be operating: an excess of cytosolic reducing power and a mechanism for its translation solely to the H2O2-promoted interchain disulfide bond.

The existence was recently reported in muscle of the presence of a pair of cysteine residues at N terminus of type I PKA regulatory subunits, which align in antiparallel fashion to each other and form disulfide bonds linking the Cys-17 and -38 (in rat) of different RI proteins (21). In contrast to the effects described herein with respect to type II PKA, oxidative disulfide formation of the two RI subunits of PKA causes subcellular translocation and activation of the PKA. Notwithstanding this, because RII subunits lack N-terminal cysteine residues, it is unable to form interprotein disulfides between RII subunits and therefore cannot be activated by this mechanism. Thus, the formation of protein disulfides exerts opposite effects on type I and II PKA holoenzymes. RIIβ is predominantly expressed in

FIGURE 9. Scheme summarizing cAMP-mediated PKA blockade activation by insulin-induced H2O2 and its main metabolic consequences. In starvation or stress condition-associated hypoglycemia, adrenaline is released, and through β-adrenoreceptors in adipocytes it activates the adenylyl cyclase that increases the cAMP pool. This cyclic nucleotide activates PKA holoenzyme by separating the two catalytic from the two regulatory subunits. The active catalytic subunits catalyze perilipin and HSL phosphorylation to raise lipolysis and to release free fatty acids and glycerol from adipocytes. Opposite metabolic conditions are present under relax conditions and after feeding, which leads to physiologic hyperglycemia; insulin is liberated from pancreas, which impinges its receptors in adipose tissue to activate an NADPH oxidase and generate H2O2. Formed H2O2 in the extracellular space, with the aid of specific mammalian aquaporins (57), reaches type II PKA that has been described as being anchored to a protein kinase A-anchoring protein targeted to plasma membrane (58). Following this hypothetical route, H2O2 reacts with PKA holoenzyme and oxidizes a -SH from Cys-199 in catalytic subunit and -SH from Cys-97 in the regulatory subunit to constitute an S–S bond that impairs the cAMP activating role on PKA holoenzyme. Target proteins are not phosphorylated by active catalytic PKA subunit; lipolysis is lowered. The thioredoxin plus thioredoxin reductase system can renew -SH groups from the newly formed S–S bond, regenerating PKA holoenzyme for it to be activable again by cAMP.
tissues involved in body weight regulation (62), including liver, brain, and brown adipose and white adipose tissues, where it is the main PKA regulatory isof orm (63, 64). Although the majority of RIα-deficient mice are normal (65, 66), RIβ-deficient mice display a lean phenotype that is resistant to diet-induced obesity, increased lipolysis, and reduced insulin (63, 67).

In regard to the “insulin-like” effects of H2O2 on adipose tissue described many years ago (9, 12) and more recently confirmed in experiments in which H2O2 was generated during oxidation of amines by amino oxidases (13), data from our paper comprise an advance in understanding of the molecular basis of some of these insulin-like effects. The H2O2 snag for paper comprise an advance in understanding of the molecular oxidation of amines by amino oxidases (13), data from our catalyticitory messages: fed versus fasting; relax versus stress; insulin action versus adrenaline action; hyperglycemia versus hypoglycemia, and lipogenesis versus lipolysis. In conclusion, this is the first report demonstrating physiologic regulation of type II PKA catalytic subunit, its C199A mutant, and regulatory RII. We also thank Enrique Moreno, M.V.Z., for technical support, Alejandra Palomares for secretarial contribution, and Maggie Brunner for advice on improving the manuscript.

Acknowledgments—We thank Marietta Tsuena de Gómez Payou, Ph.D., for preparing radioactive ATP; Concepción Agundis, Ph.D., and Mohamed A. Pereyra, M.Sc., for preparing antibodies against hormone-sensitive lipase and Susan S. Taylor, Ph.D., for advice and comments and for kindly providing purified type II PKA, wild-type catalytic subunit, its C199A mutant, and regulatory RIα. We also thank Enrique Moreno, M.V.Z., for technical support, Alejandra Palomares for secretarial contribution, and Maggie Brunner for advice on improving the manuscript.

REFERENCES

1. Turner, S. M., Murphy, E. J., Neece, R. A., Antelo, F., Thomas, T., Agarwal, A., Go, C., and Hellerstein, M. K. (2003) Am. J. Physiol. 285, E790–E803
2. Strawford, A., Antelo, F., Christiansen, M., and Hellerstein, M. K. (2004) Am. J. Physiol. 286, E577–E588
3. Taylor, S. S., Yang, J., Wu, J., Haste, N. M., Radzio-Andzelm, E., and Anand, G. (2004) Biochim. Biophys. Acta 1697, 259–269
4. Tansley, I. T., Szalay, N., Hlavin, E. M., Kimmel, A. R., and Londo, S. (2004) JUBMB Life 56, 379–385
5. Holm, C., Langin, D., Manganiello, V., Belfrage, P., and Degerman, E. (1997) Methods Enzymol. 286, 45–67
6. Zmuda-Trzebiatowska, E., Manganiello, V., and Degerman, E. (2007) Cell. Signal. 19, 81–86
7. Zhang, J., Hopfeld, C. I., Taylor, S. S. Olefsky, J. M., and Tsien, R. Y. (2005) Nature 437, 569–573
8. Hopfeld, C. I., Dalle, S., and Olefsky, J. M. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 161–163
9. Mukherjee, S. P. (1980) Biochem. Pharmacol. 29, 1239–1246
10. Krieger-Brauer, H. I., Medda, P. K., and Kather, H. (1997) Methods Enzymol. 286, 45–67
11. Zmuda-Trzebiatowska, E., Manganiello, V., and Degerman, E. (2007) Cell. Signal. 19, 81–86
12. Zhang, J., Hopfeld, C. I., Taylor, S. S. Olefsky, J. M., and Tsien, R. Y. (2005) Nature 437, 569–573
13. Visentin, V., Prévet, D., Marti, L., and Carpenté, C. (2003) Eur. J. Pharmacol. 466, 235–243
Bioinformatics 18, 502–504
50. Mahadev, K., Motoshima, H., Wu, X., Ruddy, J. M., Arnold, R. S., Cheng, G., Lambeth, J. D., and Goldstein, B. J. (2004) Mol. Cell. Biol. 24, 1844–1854
51. Chijiwa, T., Mishima, A., Hagiwara, M., Sano, M., Hayashi, K., Inoue, T., Naito, K., Toshioka, T., and Hidaka, H. (1990) J. Biol. Chem. 265, 5267–5272
52. Enserink, J. M., Christensen, A. E., de Rooy, J., van Triest, M., Schwede, F., Genieser, H. G., Deskeland, S. O., Blank, J. L., and Bos, J. L. (2002) Nat. Cell Biol. 4, 901–906
53. Margoliash, E., Novogrodsky, A., and Schejter, A. (1960) Biochem. J. 74, 349–350
54. Vigil, D., Blumenthal, D. K., Heller, W. T., Brown, S., Canaves, J. M., Taylor, S. S., and Trewella, J. (2004) J. Mol. Biol. 337, 1183–1194
55. Zhao, J., Hoye, E., Boylan, S., Walsh, D. A., and Trewella, J. (1988) J. Biol. Chem. 273, 30448–30459
56. Powis, G., and Montfort, W. R. (2001) Annu. Rev. Pharmacol. Toxicol. 41, 261–295
57. Gagelmann, M., Reed, J., Kübler, D., Pyerin, W., and Kinzel, V. (1980) Proc. Natl. Acad. Sci. U. S. A. 77, 2492–2496
58. Blaschke, R. J., Monaghan, A. P., Bock, D., and Rappold, G. A. (2000) Genomics 64, 187–194
59. Bienert, G. P., Møller, A. L. B., Kristiansen, K. A., Schulz, A., Møller, I. M., Schoerring, J. K., and Jahn, T. P. (2007) J. Biol. Chem. 282, 1183–1192
60. Wong, W., and Scott, J. D. (2004) Nat Rev. Mol. Cell Biol. 5, 959–970
61. First, E. A., and Taylor, S. S. (1984) J. Biol. Chem. 259, 4011–4014
62. Skalhegg, B. S., and Tasken, K. (2000) Front. Biosci. 5, D678–D693
63. Newman, K. J., Cummings, D. E., Nolan, M. A., and McKnight, G. S. (2005) Mol. Endocrinol. 19, 982–991
64. Cummings, D. E., Brandon, E. P., Planas, J. V., Motamed, K., Idzerda, R. L., and McKnight, G. S. (1996) Nature 382, 622–626
65. Burton, K. A., Johnson, B. D., Hausken, Z. E., Westonbroek, R. E., Idzerda, R. L., Scheuer, T., Scott, J. D., Catterall, W. A., and McKnight, G. S. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 11067–11072
66. Burton, K. A., Treash–Osiolo, B., Muller, C. H., Dunphy, E. L., and McKnight, G. S. (1999) J. Biol. Chem. 274, 24131–24136
67. Schreyer, S. A., Cummings, D. E., McKnight, G. S., and LeBoeuf, R. C. (2001) Diabetes 50, 2555–2562
68. Humphries, K. M., Pennypacker, J. K., and Taylor, S. S. (2007) J. Biol. Chem. 282, 22072–22079
69. Niedner, R. H., Buzko, O. V., Haste, N. M., Taylor, A., Gribskov, M., and Taylor, S. S. (2006) Proteins 63, 78–86