THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 282, NO. 30, pp. 22072–22079, July 27, 2007


# Redox Regulation of cAMP-dependent Protein Kinase Signaling

## *KINASE VERSUS PHOSPHATASE INACTIVATION**

Received for publication, March 26, 2007, and in revised form, May 31, 2007 Published, JBC Papers in Press, June 4, 2007, DOI 10.1074/jbc.M702582200

**Kenneth M. Humphries, Juniper K. Pennypacker, and Susan S. Taylor**[1]

*From the Howard Hughes Medical Institute, Department of Chemistry and Biochemistry and Department of Pharmacology, University of California, San Diego, La Jolla, California 92093-0654*

**Many components of cellular signaling pathways are sensitive to regulation by oxidation and reduction. Previously, we described the inactivation of cAMP-dependent protein kinase (PKA) by direct oxidation of a reactive cysteine in the activation loop of the kinase. In the present study, we demonstrate that in HeLa cells PKA activity follows a biphasic response to thiol oxidation. Under mild oxidizing conditions, or short exposure to oxidants, forskolin-stimulated PKA activity is enhanced. This enhancement was blocked by sulfhydryl reducing agents, demonstrating a reversible mode of activation. In contrast, forskolin-stimulated PKA activity is inhibited by more severe oxidizing conditions. Mild oxidation enhanced PKA activity stimulated by forskolin, isoproterenol, or the cell-permeable analog, 8-bromo-cAMP. When cells were lysed in the presence of serine/threonine phosphatase inhibitor, NaF, the PKA-enhancing effect of oxidation was blunted. These results suggest oxidation of a PKA-counteracting phosphatase may be inhibited, thus enhancing the apparent kinase activity. Using an *in vivo* PKA activity reporter, we demonstrated that mild oxidation does indeed prolong the PKA signal induced by isoproterenol by inhibiting counteracting phosphatase activity. The results of this study demonstrate in live cells a unique synergistic mechanism whereby the PKA signaling pathway is enhanced in an apparent biphasic manner.**

cAMP-dependent protein kinase (PKA or cAPK)[2] is a ubiquitously expressed kinase, critical for diverse cellular functions (1). Regulation of the kinase is primarily achieved by the production and degradation of the second messenger cAMP in response to extracellular stimuli, including hormones and neurotransmitters (2). Binding of these ligands to $G_s$-coupled receptors initiates the activation of adenylate cyclase and production of cAMP from ATP. PKA normally exists as an inactive heterotetramer composed of two regulatory subunits and two catalytic subunits (3–5). Binding of cAMP to the regulatory subunits induces the release of the active catalytic subunit, initiating the phosphorylation of numerous downstream substrates, including ion channels at the plasma membrane and sarcoplasmic reticulum (1, 6), the transcription factor cAMP-response element-binding protein in the nucleus (1, 7–9), and phosphorylase kinase in the cytosol (1, 10). The PKA signal persists until cAMP is hydrolyzed by phosphodiesterases (2) or the free catalytic subunit is inhibited by the heat-stable inhibitor PKI (11). Upon inactivation of PKA by the removal of cAMP, the phosphorylated substrate will persist until reversed by phosphatase activity.

Reactive oxygen species are capable of modulating the response of numerous cell signaling pathways (12–15), including that of PKA (16–21). Oxidants can potentially regulate the PKA pathway at multiple points, including the production and degradation of cAMP, and the phosphorylation and dephosphorylation of substrates. At the level of cAMP production, biochemical evidence has demonstrated that hydrogen peroxide has a biphasic effect upon adenylate cyclase activity in rat heart membranes, with activity increasing upon brief exposure to oxidants, whereas inhibition is observed upon longer exposure (19). Furthermore, perfusion of isolated rat hearts with a high concentration of $H_2O_2$ was found to block the stimulation of cAMP by forskolin, a potent activator of adenylate cyclase (18). In contrast, treatment of cells in culture with hydrogen peroxide enhances forskolin-dependent activation of adenylate cyclase activity via a tyrosine phosphorylation-dependent mechanism (20–23).

Oxidation can also regulate the phosphorylation and dephosphorylation of protein substrates. Previously, we demonstrated that site-specific oxidation of the catalytic subunit of PKA occurs via oxidation of $Cys^{199}$ in the kinase activation loop (16). Oxidation of this site via the formation of a protein-glutathione mixed disulfide inhibited kinase activity and primed a critical phosphorylation site in the activation loop, $pThr^{197}$, for dephosphorylation (17). Deglutathionylation and reactivation of the kinase is achieved by treatment with reducing agents or the protein-glutathione reducing enzyme glutaredoxin (24). In contrast, reactive oxygen species could potentially enhance the persistence of the PKA cascade via inhibition of phosphatases. Treatment of cells in culture enhances threonine phosphorylation via glutathionylation and inhibition of PP2A (25). Cal-

* This work was supported by National Institutes of Health Grant GM19301. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] To whom correspondence should be addressed: Dept. of Chemistry and Biochemistry, The University of California, 9500 Gilman Dr., Leichtag Bldg., San Diego, La Jolla, CA 92093-0654. Tel.: 858-534-3677; Fax: 858-534-8193; E-mail: staylor@ucsd.edu.

[2] The abbreviations used are: PKA, cAMP-dependent protein kinase; DTT, dithiothreitol; IBMX, isobutylmethylxanthine; OA, okadaic acid; FRET, fluorescence resonance energy transfer; CFP, cyan fluorescent protein; YFP, yellow fluorescent protein; 8-Br-cAMP, 8-bromo-cAMP; PP1 and PP2A, phosphatases 1 and 2A; AKAR, A-kinase activity reporter.

cineurin has also been reported to be sensitive to $H_2O_2$-mediated inactivation in a thiol-oxidation-dependent manner (26, 27). The relative sensitivity of PKA and phosphatases to inhibition by thiol oxidation and reactivation by reduction suggests potentially critical points of redox regulation.

In the present study, we demonstrate the balance between kinase and phosphatase oxidation in regulating the PKA phosphorylation signal. Incubating HeLa cells with increasing concentrations of the cell-permeable, sulfhydryl-specific oxidant, diamide, induced an apparent biphasic effect upon phosphorylation of PKA substrate. Brief or a low concentration of diamide enhanced phosphorylation induced by the PKA agonists forskolin, isoproterenol, or 8-Br-cAMP. In contrast, longer or higher concentrations of diamide inhibited PKA phosphorylation induced by these same agonists. *In vivo* monitoring of PKA activity using a genetically encoded FRET reporter confirmed that mild oxidation enhances the duration of substrate phosphorylation via inhibition of phosphatase activity.

## EXPERIMENTAL PROCEDURES

*Materials*—All chemicals and reagents were obtained from Sigma-Aldrich unless otherwise noted.

*PKA Oxidation Studies*—HeLa cells (ATCC) were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (CellGro) containing penicillin and streptomycin. Prior to treatment, cells (~75% confluent) were washed with phosphate-buffered saline and then placed in serum-free Dulbecco's modified Eagle's medium. Cells were incubated with indicated concentrations of forskolin, isoproterenol, or 8-Br-cAMP, and then incubated for 10 min at 23 °C. Indicated concentrations of diamide were then added to the cells for 30 min, or stated otherwise, and then allowed to incubate for indicated times at 23 °C. Cells were then washed with phosphate-buffered saline, scraped, and collected in 1.0 ml of phosphate-buffered saline, and pelleted by spinning 5 min at 500 × *g*. Cell pellets were then resuspended in 0.5 ml of phosphate-buffered saline and spun again. Pellets were then lysed by resuspending the pellet in 200 μl of a buffer containing 150 mM NaCl, 50 mM Tris, pH 7.2, and 1.0% Nonidet P-40 (Buffer B) with DTT, NaF, or the indicated amounts of okadaic acid (EMD Biosciences) or FK506 (Sigma). After incubating on ice for 10 min, the lysate was spun for 5 min at 9300 × *g*, and the soluble fraction was collected. Western blot analysis indicated nearly complete solubilization of PKA under these conditions. Protein concentrations of cell lysates were determined using Coomassie Blue Protein Assay Reagent (Pierce) with bovine serum albumin as the standard.

*PKA Activity*—PKA activity is determined using a non-radioactive PKA assay system (16, 28) utilizing fluorescently labeled Kemptide (Caliper), the prototypical PKA substrate. In the absence of phosphorylation the substrate has no net charge. Upon phosphorylation, the peptide substrate has a net negative charge. For this assay, 7.0 μg of cell lysate was added to a reaction mixture containing 1.0 mM ATP, 5.0 mM $MgCl_2$, and 25 mM Tris, pH 7.2, in the presence or absence 5.0 μM cAMP for 10–30 min, and then stopped by the addition of the PKA-specific inhibitor PKI (50 μM final). Negatively charged, phosphorylated peptide is then separated from neutral, uncharged peptide by agarose (0.8%) gel electrophoresis run at 100 V for 25 min followed by UV imaging analysis. All kinase activities are determined to be both cAMP-dependent and PKI-sensitive as well as linear within the time frame of the reaction.

*Statistical Analysis*—Statistical analyses were performed using unpaired Student's *t* test, with $p < 0.05$ differences considered significant. Values are expressed as means ± S.E. of means.

*Constructs*—The cAMP-dependent protein kinase activity reporter (AKAR2.2) was a generous gift of Dr. Jin Zhang (Johns Hopkins University) and has been previously described (29). Briefly, this construct contains a YFP (YFP), cyan fluorescent protein (CFP), a PKA-specific phosphorylation site, and a forkhead-associated domain. Phosphorylation of the substrate site induces binding to the forkhead-associated domain and causes a subsequent increase in FRET intensity.

*Imaging*—Cells were imaged in the dark at room temperature, with forskolin or diamide added as indicated. Images were acquired on a Zeiss Axiovert microscope (Carl Zeiss Microimaging, Inc.) using a MicroMax digital camera (Roper-Princeton Instruments) controlled by MetaFluor 3.0 software (Universal Imaging, West Chester, PA). Data were collected through a 10% neutral density filter. Yellow fluorescent protein images were acquired through a 495/10 nm excitation filter, a 505 nm dichroic mirror, and a 568/55 nm emission filter. CFP images were obtained through a 420/10 nm excitation filter, a 450 nm dichroic mirror, and a 475/40 nm emission filter. FRET images were obtained through the same excitation filter, and dichroic mirror as CFP images, but the emission filter was 535/25 nm. The integration time was 100 ms.

## RESULTS

*PKA Phosphorylation Follows a Biphasic Response to Increasing Oxidation*—As previously described, PKA is inhibited by oxidation via either glutathionylation of $Cys^{199}$ in the activation loop, or the formation of an internal disulfide bond between $Cys^{199}$ and $Cys^{343}$ (16). However, in HeLa cells treated with a low concentration of adenylate cyclase activator, forskolin (10 μM), and increasing concentrations of the sulfhydryl specific oxidant, diamide, there is a biphasic response in apparent PKA activity. As shown in Fig. 1*A*, following treatment of cells with concentrations of diamide up to 100 μM, PKA activity in cell lysates was enhanced in a linear manner. Kinase activity nearly doubled following treatment of cells with 100 μM diamide (control activity of 595 ± 43.6 *versus* 1022 ± 103.6 pmol × $min^{-1}$ × $mg^{-1}$ following 100 μM diamide treatment). In contrast, and consistent with previous results (16), concentrations of diamide above 100 μM inhibited PKA activity in a concentration-dependent manner. Incubation of cells with 2.0 mM diamide maximally reduced PKA activity to 326 ± 74.1 pmol × $min^{-1}$ × $mg^{-1}$.

Diamide is a strong sulfhydryl-specific oxidant that preferentially oxidizes small thiol-containing molecules and promotes protein glutathionylation (30). In addition, diamide can catalyze the formation of intra- and intermolecular disulfide bonds via oxidation of protein cysteines. As shown in Fig. 1*B*, inclusion of DTT (25 mM) to cell lysates reversed the enhanced PKA phosphorylation induced by 100 μM diamide. This result dem-

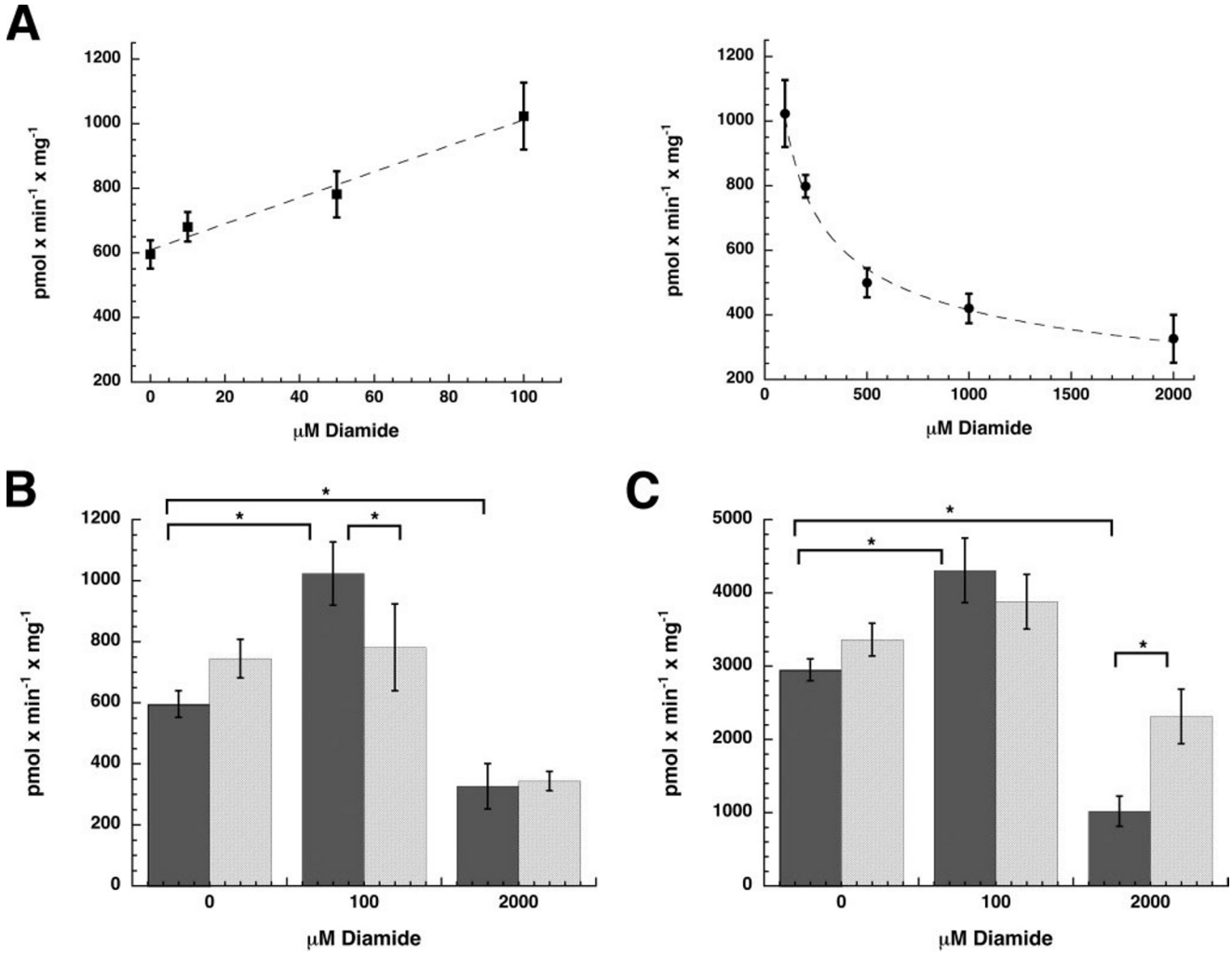


FIGURE 1. **Forskolin-stimulated PKA activity follows a biphasic response to an increasing concentration of diamide.** *A*, HeLa cells were incubated with 10 $\mu$M forskolin for 10 min at 23 °C prior to the addition of indicated amounts of diamide. *B*, following incubation with indicated amounts of diamide, HeLa cells were lysed in the presence (*light gray*) or absence (*dark gray*) of 25 mM DTT. *C*, HeLa cells were incubated with 50 $\mu$M forskolin and 100 $\mu$M IBMX for 10 min at 23 °C prior to the addition of indicated amounts of diamide, and then either lysed in the presence (*light gray*) or absence (*dark gray*) of 25 mM DTT. For all experiments, cells were incubated with indicated diamide concentrations for 30 min at 23 °C, collected, and lysed, protein concentrations were standardized, and PKA activity was determined as described under "Experimental Procedures." Values are means ± S.E. ($n = 5$); *, significant difference between indicated sample treatments ($p < 0.05$).

onstrates that the activity-enhancing effect that diamide had on PKA phosphorylation is dependent upon the oxidation state of sulfhydryls. In contrast, DTT was unable to recover the inhibition of PKA at 2.0 mM diamide.

To characterize the mechanism whereby oxidation enhances PKA activity, we next examined the ability of diamide to enhance phosphorylation under cAMP-saturating conditions. It was hypothesized that, if a low concentration of diamide enhances adenylate cyclase activity, the effect it has on PKA substrate phosphorylation would be blocked under conditions of high cellular cAMP in which the total pool of PKA was activated. Using a high concentration of forskolin (50 $\mu$M) and including a phosphodiesterase inhibitor, IBMX (100 $\mu$M), induced a 6-fold increase in PKA activity as compared with 10 $\mu$M forskolin alone (Fig. 1*C*). PKA activity with 50 $\mu$M forskolin and 100 $\mu$M IBMX was 2948.9 ± 148 *versus* 595 ± 43.6 pmol × $min^{-1}$ × $mg^{-1}$ for cells treated with 10 $\mu$M forskolin. This increase in activity was not further enhanced by including cAMP (5.0 mM) to reactions, indicating the entire pool of kinase was activated under these conditions (data not shown). Nevertheless, incubation of cells with 100 $\mu$M diamide increased PKA activity under these experimental conditions but was less dramatic than seen with low (10 $\mu$M) forskolin concentrations (Fig. 1*C*). As shown, 100 $\mu$M diamide increased activity by 31% in high forskolin/IBMX-treated cells as compared with a 72% increase in activity with low forskolin treatment. The biphasic effect of increasing diamide concentrations was also seen under maximal PKA stimulation, with 2.0 mM diamide decreasing forskolin/IBMX-induced PKA activity by 66%. Inclusion of DTT in the cell lysates was able to partially lower kinase activity enhanced by 100 $\mu$M diamide and largely recovered activity induced by 2.0 mM diamide (Fig. 1*C*). These results suggest that oxidation is not enhancing PKA activity through increased adenylate cyclase activity and that the effect diamide is having on phosphorylation of the peptide substrate is thiol-oxidation-dependent.

To further characterize the effect of oxidation on PKA substrate phosphorylation, we next looked at the effect of 1.0 mM

diamide, a concentration that inhibited PKA activity at 30 min (Fig. 1*A*), with increasing time. As shown in Fig. 2, incubation of cells with 10 $\mu$M forskolin and 1.0 mM diamide resulted in an immediate enhancement of PKA activity with decreasing activity over time. Diamide added just prior to cell lysis had a PKA activity of 1137 ± 235 pmol × $min^{-1}$ × $mg^{-1}$ as compared with 595 ± 43 pmol × $min^{-1}$ × $mg^{-1}$ for those that were treated with forskolin alone. Activity peaked after 10 min, increasing to 2537 ± 407 pmol × $min^{-1}$ × $mg^{-1}$, and then decreasing over the next 20 min to below that of the zero time. The effect of diamide on PKA activity was dependent upon the oxidation state of sulfhydryls. Addition of DTT (25 mM) to the cell lysates largely reversed the enhanced PKA activity seen by short durations of exposure to diamide.

*Diamide Enhances Receptor-mediated and Direct Activation of PKA*—Physiological activation of PKA normally proceeds through a pathway whereby an extracellular ligand binds a G protein-coupled receptor. Activation of a $G_s$ type G protein is followed by downstream activation of adenylate cyclase and production of cAMP. The amount of cAMP available for PKA activation is in turn regulated by phosphodiesterases. The phosphorylation of substrates is reversed by serine/threonine protein phosphatases. Thus, there are several regulatory points at which the observed PKA activity can be redox-regulated. The results in Fig. 1*B* suggested that the observed enhanced PKA activity was not a result of increased adenylate cyclase activity. We therefore next looked at the ability of thiol oxidation to enhance PKA activity with either isoproterenol, a $\beta$-adrenergic agonist, or 8-Br-cAMP, a cell-permeable cAMP analog.

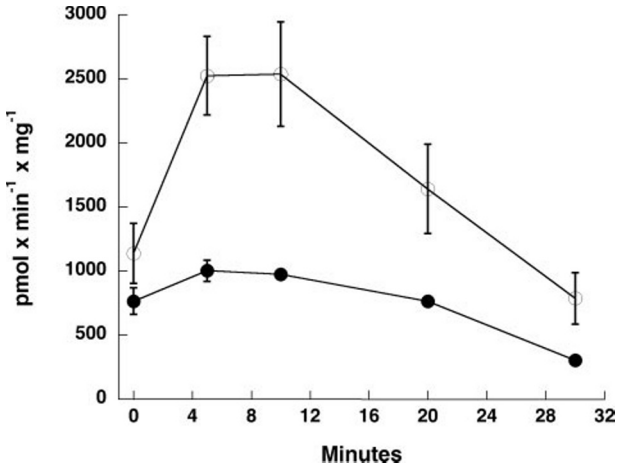


FIGURE 2. **Forskolin-stimulated PKA activity follows a biphasic response to duration of exposure to diamide.** HeLa cells were incubated with 10 $\mu$M forskolin for 10 min at 23 °C prior to the addition of 1.0 mM diamide. Cells were incubated with diamide for the indicated times, collected, and lysed in either the presence (*close circles*) or absence (*open circles*) of 25 mM DTT, protein concentrations were standardized, and PKA activity was determined as described under "Experimental Procedures." Values are means ± S.E. ($n$ = 5).

As shown in Table 1, HeLa cells treated with isoproterenol (5 $\mu$M) and IBMX (100 $\mu$M) had a PKA activity of 695 ± 62 pmol × $min^{-1}$ × $mg^{-1}$ as compared with 119 ± 18 nmol × $min^{-1}$ × $mg^{-1}$ for untreated cells or cells that were incubated with only isoproterenol (data not shown). Treatment of cells with diamide alone (100 $\mu$M for 30 min) had minimal effect on PKA activity (218 ± 80 pmol × $min^{-1}$ × $mg^{-1}$) indicating diamide is not enhancing PKA activity by direct activation of the kinase or adenylate cyclase. However, this concentration of diamide significantly enhanced the isoproterenol/IBMX-induced activation of PKA, doubling the activity to 1164 ± 96 nmol × $min^{-1}$ × $mg^{-1}$. Furthermore, this enhancement was reversed by addition of 25 mM DTT to the lysis buffer, reducing the activity to 815 ± 122 pmol × $min^{-1}$ × $mg^{-1}$, indicating that oxidized sulfhydryls contribute to the observed PKA activity. Because diamide enhanced PKA activity even in the presence of excess phosphodiesterase inhibitor, IBMX, it was concluded that diamide is not enhancing PKA activity by inhibiting the breakdown of cAMP.

We next looked at the ability of diamide to enhance the direct activation of PKA by the cell-permeable analog 8-Br-cAMP. As shown in Table 1, a low concentration of 8-Br-cAMP (10 $\mu$M) significantly enhanced PKA activity (807 ± 61 pmol × $min^{-1}$ × $mg^{-1}$) as compared with no treatment (102 ± 17 pmol × $min^{-1}$ × $mg^{-1}$). Treatment of cells with diamide (100 $\mu$M) and 10 $\mu$M 8-Br-cAMP significantly enhanced the activation of PKA to 1316 ± 86 pmol × $min^{-1}$ × $mg^{-1}$. Inclusion of DTT to the cell lysate reversed this enhancement but had no effect on control activity (Table 1). This result conclusively shows that oxidation is not enhancing PKA activity upstream of cAMP production.

*The Effect of Diamide and Phosphatase Inhibitors on PKA Activity Are Not Additive*—Our results indicate that oxidation by low diamide concentrations enhances PKA activity and is reversible by sulfhydryl reduction. This enhancement occurs at the level of apparent PKA activity and is not a result of increased cAMP production or decreased cAMP breakdown. We thus hypothesized that, under our experimental conditions, oxidation may be acting to enhance the apparent PKA activity by inhibiting a competing phosphatase.

As shown in Fig. 3, inclusion of a nonspecific phosphatase inhibitor, NaF, in cell lysis buffer had a minor effect on PKA activity in the absence of forskolin (basal activity of 132 ± 16 × $min^{-1}$ × $mg^{-1}$ *versus* 184 ± 4 pmol × $min^{-1}$ × $mg^{-1}$ with NaF). However, cell cultures treated with forskolin (10 $\mu$M) and then lysed in the presence of NaF showed a dramatic increase in the observed PKA activity (1373 ± 73 pmol × $min^{-1}$ × $mg^{-1}$), as compared with forskolin alone (621 ± 88 pmol × $min^{-1}$ × $mg^{-1}$). This result shows that, under conditions of limited cAMP production, the persistence of PKA substrate phosphorylation in cell lysates is dictated by phosphatase activity. Dia-

**TABLE 1**

**Oxidation enhances PKA phosphorylation of a peptide substrate and is reversible by thiol reduction**

HeLa cells were treated with PKA agonists and 100 $\mu$M diamide, as indicated, for 30 min at 23 °C and PKA activity assayed as described under "Experimental Procedures." Values are expressed as pmol × $min^{-1}$ × $mg^{-1}$ of cell lysate and represent the means ($n$ = 5) ± S.E.

| | No addition | PKA agonist | PKA agonist plus DTT | PKA agonist plus diamide | PKA agonist plus diamide plus DTT |
|---|---|---|---|---|---|
| | | | *pmol × min⁻¹ × mg⁻¹* | | |
| Isoproterenol plus IBMX | 119.1 ± 18 | 695.92 ± 62 | 787 ± 93 | 1184 ± 96 | 849 ± 121 |
| 8-Br-cAMP | 102 ± 17 | 798 ± 62 | 813 ± 102 | 1316 ± 86 | 914 ± 144 |

mide (100 $\mu$M) enhanced forskolin-stimulated PKA activity ($1074 \pm 79$ pmol $\times$ $min^{-1} \times mg^{-1}$) to a lesser extent than NaF treatment. Cells treated with forskolin and diamide and then lysed in buffer containing NaF had an activity of $1503 \pm 60$ $min^{-1} \times mg^{-1}$. This result demonstrates that diamide and NaF treatment both enhance PKA activity to a similar extent, and that their effects upon the observed PKA activity are non-additive. This suggests that oxidation is enhancing the apparent PKA activity by inactivating a phosphatase(s) that dephosphorylates PKA substrates.

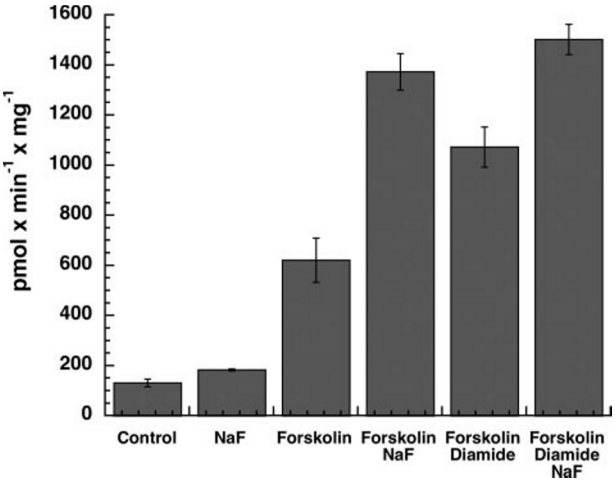


FIGURE 3. **Diamide and NaF enhance PKA activity in a non-additive manner.** HeLa cells were incubated with 10 $\mu$M forskolin for 10 min at 23 °C prior to the addition of 100 $\mu$M diamide. Cells were incubated an additional 30 min at 23 °C, collected, lysed in either the presence or absence of 25 mM NaF, as indicated, protein concentrations were standardized, and PKA activity was determined as described under "Experimental Procedures." Values are means $\pm$ S.E. ($n = 5$).

Phosphorylated PKA substrates are dephosphorylated by numerous phosphatases, including PP1 (8), PP2A (31), PP2C (32), and calcineurin (also known as PP2B) (33–36). Phosphatase identity can be characterized by drastically different sensitivities to inhibition by okadaic acid (OA): the $K_i$ for PP2A is 30 pM, the $K_i$ for PP1 is 150 nM, and the $K_i$ for calcineurin is 4–5 $\mu$M (37–39). PP2C is insensitive to OA (39, 40). We determined that concentrations of OA in the picomolar range (added to the lysis buffer) had no enhancing effect on PKA activity in cells treated with 10 $\mu$M forskolin. In contrast, 100 nM OA enhanced apparent PKA activity by 30.0% $\pm$ 7.5%. PKA activity was further enhanced by 1 $\mu$M OA, with activity 45.4% $\pm$ 4.1% higher than treatment with 10 $\mu$M forskolin alone. Increasing OA to 3.0 $\mu$M did not further increase apparent PKA activity. FK506 (1.0 mM, added prior to lysis), an inhibitor of calcineurin (41, 42), had no enhancing effect on PKA activity.

Sensitivity to OA suggests PP1 is contributing to dephosphorylation of PKA substrate. However, OA is only able to enhance PKA activity by 45.4%, whereas the nonspecific phosphatase inhibitor NaF is able to enhance activity by 121%. It is therefore likely that more than one phosphatase is contributing to dephosphorylation. For example, PP2C, the least understood phosphatase, is known to be activated by magnesium or manganese, but has no known natural regulators or inhibitors (43, 44) except NaF (40). Magnesium is required for PKA activity and is included in all assays. Thus, under conditions in which PKA is assayed, it would be expected PP2C is also activated.

*In Vivo Enhancement of PKA Signal by Oxidation*—The above biochemical characterization suggests that the duration of the PKA phosphorylation signal can be regulated reversibly by thiol oxidation. This mechanism may represent an important means of regulating the duration of PKA signaling *in vivo* and was, therefore, assessed using live cell imaging. A genetically encoded PKA reporter, AKAR2.2, was transiently transfected into HeLa cells. This reporter is specifically phosphorylated by PKA, resulting in a measurable change in FRET (29, 45, 46). As shown in Fig. 4, treatment of cells expressing the reporter with isoproterenol (5.0 $\mu$M) resulted in a transient phosphorylation event. Dephosphorylation, and reversal of FRET, occurred without washing away the isoproterenol. The FRET response, however, could be recovered by addition of IBMX (100 $\mu$M) after the isoproterenol (Fig. 4). IBMX did not induce phosphorylation of the reporter without pretreatment of cells with isoproterenol (data not shown). This experiment demonstrates the

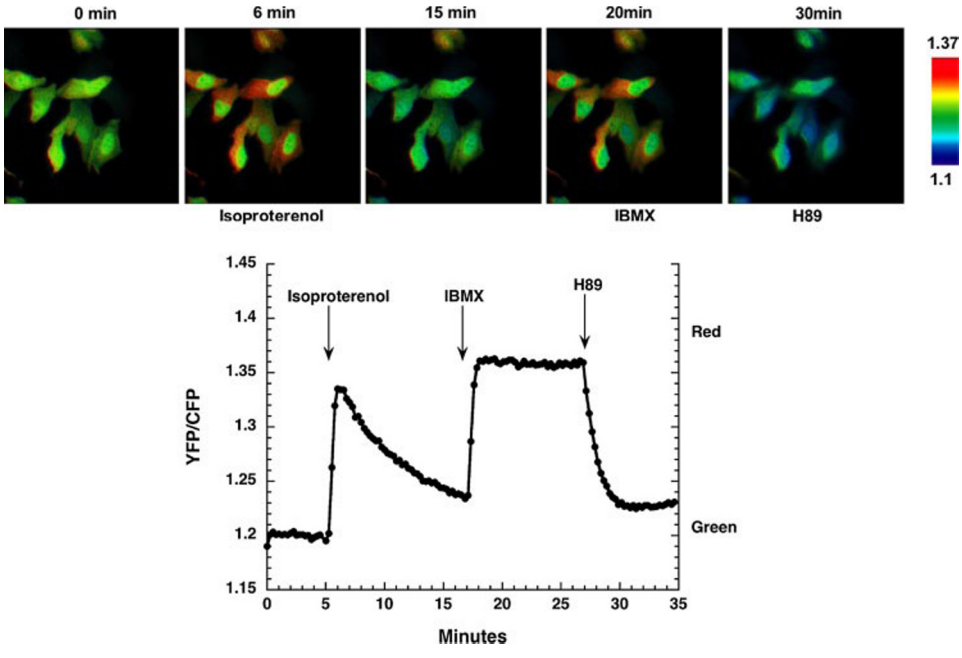


FIGURE 4. **PKA activity is transiently stimulated *in vivo* by isoproterenol.** HeLa cells were transfected with AKAR2.2 plasmid, as described under "Experimental Procedures," and imaged 12–16 h later. *Top*, images are presented in false color, with relative FRET intensities represented by the indicated *color scale*. *Bottom*, this tracing represents a typical, single cell measurement taken from the shown field, with the *y*-axis indicating the ratio of fluorescence emission at the YFP (568 nm) *versus* CFP (475 nm) wavelength using 420 nm excitation. Images were obtained every 15 s. Isoproterenol (5.0 $\mu$M), IBMX (100 $\mu$M), and H89 (50 $\mu$M) were added to the dish at 5.0, 17, and 27 min, respectively.

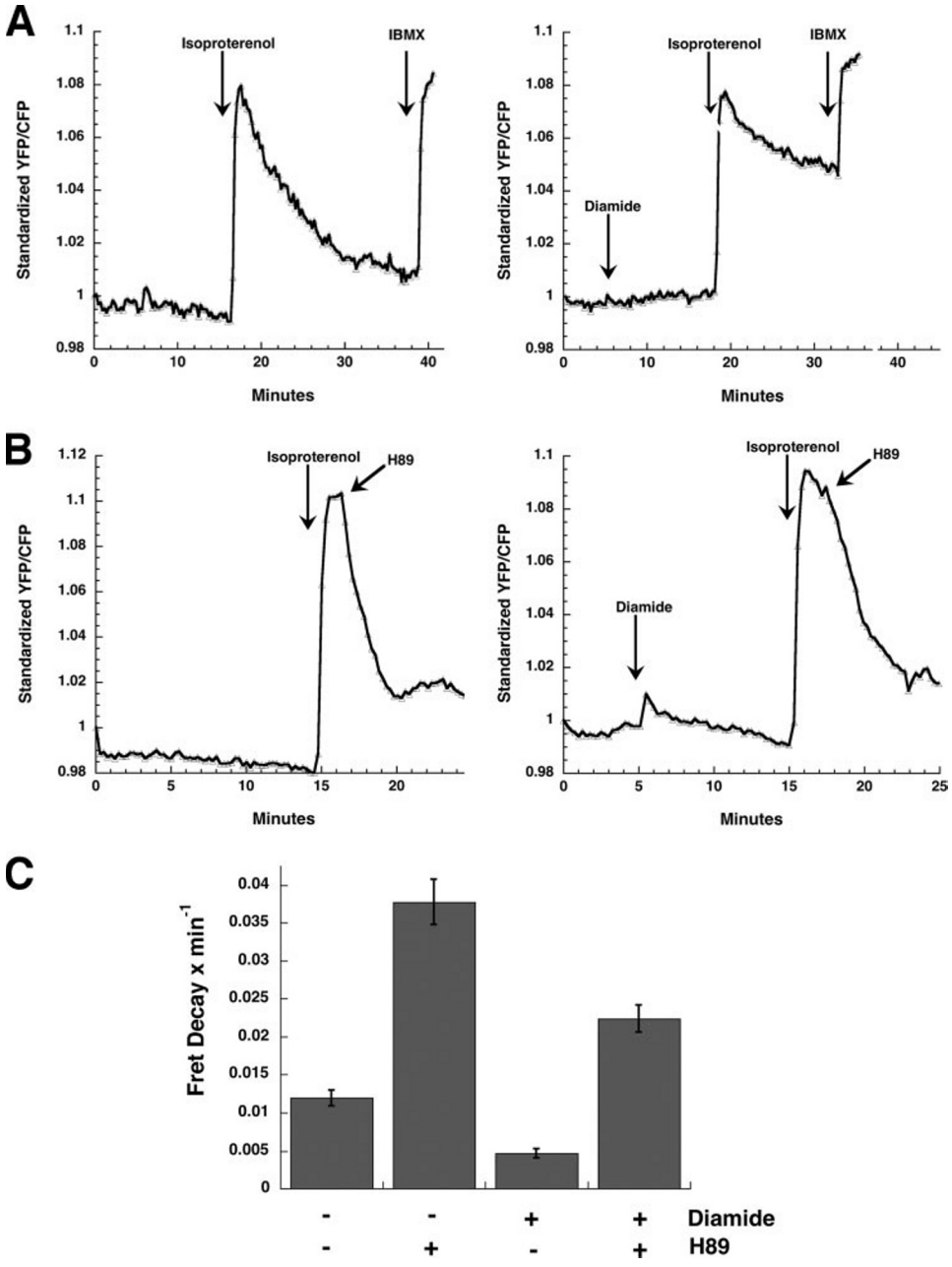


FIGURE 5. **Diamide enhances the isoproterenol-mediated PKA signal by decreasing the rate of substrate dephosphorylation.** HeLa cells were transfected with AKAR2.2 plasmid, as described under "Experimental Procedures," and imaged 12–16 h later. The *tracings* represent a typical, single cell measurement, with the *y*-axis indicating the ratio of fluorescence emission at the YFP (568 nm) *versus* CFP (475 nm) wavelength using 420 nm excitation. Images were obtained every 15 s. *A*, cells were incubated with diamide (100 $\mu$M, *right panel)* or without (*left panel*) for 10 min prior to the addition of 5.0 $\mu$M isoproterenol at 15 min. After decay of the FRET ratio, IBMX (100 $\mu$M) was added, and the signal was recovered. *B*, cells were incubated as in *A*, but H89 (50 $\mu$M) was added 2.0 min after the addition of isoproterenol. *C*, the rate of decay of the FRET ratio following the addition of isoproterenol was calculated as a linear slope. Values are means ± S.E. ($n$ = 3 experiments, and a minimum of 6 cells for each condition).

dynamic nature of receptor-mediated PKA signaling *in vivo*, with phosphodiesterase activity limiting the duration of a PKA signal.

Fig. 5*A* compares the isoproterenol response of control cells *versus* cells treated with diamide (100 $\mu$M for 10 min). Although control cells had a transient phosphorylation and dephosphorylation of AKAR2.2, the FRET response in cells treated with diamide was more prolonged. The time from isoproterenol addition to AKAR phosphorylation was not affected by diamide pretreatment, nor was the relative FRET increase (Fig. 5*A*). However, the relative rate of dephosphorylation, as calculated by measuring the rate of FRET decay, was significantly slower. Fig. 5*C* quantitatively shows that, following isoproterenol stimulation, the rate of FRET decay in diamide-treated cells (0.0046 ± 0.0001 FRET units × $min^{-1}$) was about one-third the rate of control cells (0.0119 ± 0.0011 FRET units × $min^{-1}$).

The rate of AKAR dephosphorylation following isoproterenol stimulation is controlled by the activity of PKA and an opposing phosphatase. Addition of an inhibitor of PKA, H89 (50 $\mu$M), causes the rapid dephosphorylation of the reporter (Fig. 5). As shown in Fig. 5*B* and quantified in Fig. 5*C*, if H89 is added immediately after addition of isoproterenol, the AKAR substrate is dephosphorylated approximately three times faster than in control cells (0.0377 ± 0.0029 *versus* 0.0119 ± 0.0011 FRET units × $min^{-1}$). We hypothesized that if diamide was inhibiting a PKA-counter acting phosphatase, the increased rate of AKAR dephosphorylation induced by H89 should be attenuated. Cells were pretreated with diamide (100 $\mu$M for 10 min) prior to the addition of isoproterenol. Addition of H89 (50 $\mu$M) immediately after the addition of isoproterenol increased the rate of FRET decay in oxidized cells but was slower than non-oxidized samples (0.0223 ± 0.0017 in diamide-treated cells *versus* 0.0377 ± 0.0029 in control cells, Fig. 5*C*). This result conclusively demonstrates that, in PKA inhibited cells, diamide inactivates a phosphatase that dephosphorylates PKA substrates and leads to an altered PKA signal *in vivo*.

## DISCUSSION

PKA is a ubiquitously expressed signaling molecule, capable of phosphorylating numerous downstream substrates. Exquisite regulation of kinase activity is essential for maintaining downstream specificity initiated by discrete stimuli. Previously, we had demonstrated that, in addition to classic mechanisms of

kinase regulation, the catalytic subunit of PKA is highly sensitive to reversible, oxidation-mediated inactivation. The presence of a highly reactive cysteine ($Cys^{199}$) in the activation loop renders the kinase susceptible to the formation of a protein-glutathione-mixed disulfide (16), inactivating the kinase and enhancing dephosphorylation of the catalytic subunit by phosphatases (17). As a potentially critical mechanism of kinase regulation under normal homeostasis and during conditions of increased oxidative stress, it was critical to further explore the role of sulfhydryl oxidation in modulating kinase activity.

For these studies, we looked at the ability of the cell-permeable, sulfhydryl-specific oxidant diamide (30) to modulate PKA activity. Treatment of cells with an increasing amount of diamide had a biphasic effect on the apparent PKA activity stimulated by forskolin, 8-Br-cAMP, or isoproterenol/IBMX. Diamide in the absence of a PKA agonist had no significant effect on kinase activity, and was therefore not directly activating the kinase such as by inducing disassociation of the catalytic subunits from the regulatory subunits (47). The inhibition of PKA activity by diamide at high concentrations was expected, because under these conditions the kinase forms a protein-glutathione-mixed disulfide, reversible by DTT (Fig. 1*C*) (16, 17). However, the mechanism, whereby low concentrations of diamide or brief exposure to high concentrations of diamide enhanced PKA activity, was not immediately evident.

Oxidation is capable of enhancing PKA activity via indirect routes. It has been previously shown that hydrogen peroxide promotes the tyrosine kinase-mediated serine phosphorylation of adenylate cyclase, isoform VI (21, 23). The phosphorylation of adenylate cyclase increases its $V_{max}$, thereby enhancing PKA activity via increased cAMP availability. Our results, however, indicate that the effect diamide is having is independent of adenylate cyclase activity. A low concentration of diamide enhanced the PKA activity stimulated by both the $\beta$-adrenergic agonist, isoproterenol, and by direct activation of the kinase with the cell-permeable cAMP analog, 8-Br-cAMP (Table 1). In contrast, purified PKA catalytic subunit is inhibited by diamide in a concentration-dependent manner (16). We therefore concluded that enhanced kinase activity in cells was likely to be mediated via an indirect mechanism downstream of cAMP production or PKA activation, such as via inhibition of a competing phosphatase. This conclusion was supported by the finding that diamide and NaF, a nonspecific phosphatase inhibitor, both enhance the apparent PKA activity to a similar extent and in a non-additive manner. It was also supported by our *in vivo* measurement of PKA activity in which diamide effectively diminished the rate of dephosphorylation of the genetically encoded AKAR reporter.

The differential sensitivity of PKA and phosphatase activity to oxidative inactivation may provide an effective means of regulating substrate phosphorylation. The relative effect oxidation has upon the duration of kinase signaling is likely to be mediated by the concentration of pro-oxidants produced and the amount of active, free catalytic subunit of PKA. For example, in the diamide concentration and time courses, DTT failed to recover the apparent PKA activity upon stimulation with low forskolin and inhibition by high diamide (Figs. 1*B* and 2). In the presence of a low concentration of PKA agonist, such as 10 $\mu$M forskolin, <20% of the total kinase pool is activated (compare specific activity of PKA in the absence of diamide in Fig. 1, *B* and *C*). High concentrations of diamide inhibit both kinase and phosphatase activities. Reversal of thiol-specific oxidation by DTT restores both activities, but under conditions of low cAMP the relative phosphatase to kinase activity is increased further.

PKA substrates are dephosphorylated by various serine/threonine phosphatases, including PP1 (8), PP2C (32), PP2A (31), and calcineurin (PP2B) (33–36). Based upon pharmacological evidence, our results suggest dephosphorylation of a peptide substrate in HeLa cell lysates is due, in part, to PP1 activity. However, because OA did not block PP1-dependent dephosphorylation as completely as NaF, it can be concluded that other phosphatases are contributing to dephosphorylation of PKA substrates. PP2C, a magnesium/manganese-dependent phosphatase, is a likely candidate.

The exact identity of the phosphatase inhibited by diamide is complicated by the fact that serine/threonine phosphatases exhibit sensitivity to oxidative inactivation (48–50). For example, pro-oxidants can prolong the phosphorylation of cAMP-response element-binding protein, a transcription factor and prototypical PKA substrate, in neurons via inhibition of calcineurin (33, 34). Calcineurin has also been shown *in vitro* to be sensitive to hydrogen peroxide-mediated inactivation (26, 51). PP1 and PP2A are inhibited by $H_2O_2$ in human fibroblasts (52). Finally, PP2B activity has been shown to be inhibited by $H_2O_2$ via a thiol oxidation mechanism in *Arabidopsis* (48, 49).

The putative redox regulation of serine/threonine phosphatases is derived from the presence of redox reactive centers. Calcineurin can be inhibited by $H_2O_2$ via oxidation of its Fe(II)-Zn(II) center, and/or formation of an intramolecular disulfide bond between cysteine side chains (50, 53, 54). PP1 contains an active site motif (Cys-*X*-*X*-Cys) similar to glutaredoxin and thioredoxin (50, 55). Such a motif would be expected to form a disulfide bond readily in the presence of diamide, and is thus a likely mechanism of inhibition. Diamide preferentially oxidizes small thiol-containing molecules such as glutathione (30), forming oxidized glutathione and protein-glutathione conjugates (16). Diamide does not oxidize cysteines to sulfenic or sulfonic acids. It should be noted that low concentrations of $H_2O_2$, a relatively mild pro-oxidant that preferentially oxidizes reactive cysteines, had similar enhancing effects on PKA activity.

It is clear that oxidation, specifically to thiols, is an important means of regulating signal transduction events (56, 57). The evidence presented here demonstrates that the ability of PKA to phosphorylate a substrate, both *in vitro* and *in vivo*, can be regulated via differential oxidation and inhibition of PKA *versus* counteracting phosphatase activity. Because PKA is ubiquitously expressed and involved in a myriad of cellular functions, the implications of this reversible mechanism of regulation are likely to have broad physiological significance.

*Acknowledgments—We thank Dr. Jin Zhang (Johns Hopkins University) for providing the AKAR2.2 plasmid and helpful discussions, and Dr. Maya Kunkel (University of California, San Diego) for assistance with microscopy.*

## REFERENCES


1. Shabb, J. B. (2001) *Chem. Rev.* **101,** 2381–2411
2. Sutherland, E. W. (1972) *Science* **177,** 401–408
3. Johnson, D. A., Akamine, P., Radzio-Andzelm, E., Madhusudan, M., and Taylor, S. S. (2001) *Chem. Rev.* **101,** 2243–2270
4. Beavo, J. A., Bechtel, P. J., and Krebs, E. G. (1974) *Proc. Natl. Acad. Sci. U. S. A.* **71,** 3580–3583
5. Beavo, J. A., Bechtel, P. J., and Krebs, E. G. (1975) *Adv. Cyclic Nucleotide Res.* **5,** 241–251
6. Danila, C. I., and Hamilton, S. L. (2004) *Biol. Res.* **37,** 521–525
7. Michael, L. F., Asahara, H., Shulman, A. I., Kraus, W. L., and Montminy, M. (2000) *Mol. Cell. Biol.* **20,** 1596–1603
8. Hagiwara, M., Alberts, A., Brindle, P., Meinkoth, J., Feramisco, J., Deng, T., Karin, M., Shenolikar, S., and Montminy, M. (1992) *Cell* **70,** 105–113
9. Hagiwara, M., Brindle, P., Harootunian, A., Armstrong, R., Rivier, J., Vale, W., Tsien, R., and Montminy, M. R. (1993) *Mol. Cell. Biol.* **13,** 4852–4859
10. Brushia, R. J., and Walsh, D. A. (1999) *Front. Biosci.* **4,** D618–D641
11. Wen, W., Harootunian, A. T., Adams, S. R., Feramisco, J., Tsien, R. Y., Meinkoth, J. L., and Taylor, S. S. (1994) *J. Biol. Chem.* **269,** 32214–32220
12. Kwon, J., Lee, S. R., Yang, K. S., Ahn, Y., Kim, Y. J., Stadtman, E. R., and Rhee, S. G. (2004) *Proc. Natl. Acad. Sci. U. S. A.* **101,** 16419–16424
13. Goldstein, B. J., Mahadev, K., Wu, X., Zhu, L., and Motoshima, H. (2005) *Antioxid. Redox Signal.* **7,** 1021–1031
14. Mahadev, K., Zilbering, A., Zhu, L., and Goldstein, B. J. (2001) *J. Biol. Chem.* **276,** 21938–21942
15. Park, H. S., Lee, S. H., Park, D., Lee, J. S., Ryu, S. H., Lee, W. J., Rhee, S. G., and Bae, Y. S. (2004) *Mol. Cell. Biol.* **24,** 4384–4394
16. Humphries, K. M., Juliano, C., and Taylor, S. S. (2002) *J. Biol. Chem.* **277,** 43505–43511
17. Humphries, K. M., Deal, M. S., and Taylor, S. S. (2005) *J. Biol. Chem.* **280,** 2750–2758
18. Persad, S., Panagia, V., and Dhalla, N. S. (1998) *Mol. Cell. Biochem.* **186,** 99–106
19. Persad, S., Elimban, V., Kaila, J., and Dhalla, N. S. (1997) *J. Pharmacol. Exp. Ther.* **282,** 1623–1631
20. Tan, C. M., Xenoyannis, S., and Feldman, R. D. (1995) *Circ. Res.* **77,** 710–717
21. Tan, C. M., Kelvin, D. J., Litchfield, D. W., Ferguson, S. S., and Feldman, R. D. (2001) *Biochemistry* **40,** 1702–1709
22. Tan, C. M., McDonald, C. G., Chorazyczewski, J., Burry, A. F., and Feldman, R. D. (1999) *Clin. Pharmacol. Ther.* **66,** 275–281
23. Ding, Q., Gros, R., Gray, I. D., Taussig, R., Ferguson, S. S., and Feldman, R. D. (2004) *Mol. Pharmacol.* **66,** 921–928
24. Shelton, M. D., Chock, P. B., and Mieyal, J. J. (2005) *Antioxid. Redox Signal.* **7,** 348–366
25. Rao, R. K., and Clayton, L. W. (2002) *Biochem. Biophys. Res. Commun.* **293,** 610–616
26. Sommer, D., Coleman, S., Swanson, S. A., and Stemmer, P. M. (2002) *Arch. Biochem. Biophys.* **404,** 271–278
27. Li, S., Wang, X., Klee, C. B., and Krieger, C. (2004) *Mol. Brain Res.* **125,** 156–161
28. MacAla, L. J., Hayslett, J. P., and Smallwood, J. I. (1998) *Kidney Int.* **54,** 1746–1750
29. Zhang, J., Hupfeld, C. J., Taylor, S. S., Olefsky, J. M., and Tsien, R. Y. (2005) *Nature* **437,** 569–573
30. Kosower, N. S., and Kosower, E. M. (1995) *Methods Enzymol.* **251,** 123–133
31. Wadzinski, B. E., Wheat, W. H., Jaspers, S., Peruski, L. F., Jr., Lickteig, R. L., Johnson, G. L., and Klemm, D. J. (1993) *Mol. Cell. Biol.* **13,** 2822–2834
32. Flajolet, M., Rakhilin, S., Wang, H., Starkova, N., Nuangchamnong, N., Nairn, A. C., and Greengard, P. (2003) *Proc. Natl. Acad. Sci. U. S. A.* **100,** 16006–16011
33. Bito, H., Deisseroth, K., and Tsien, R. W. (1996) *Cell* **87,** 1203–1214
34. Hongpaisan, J., Winters, C. A., and Andrews, S. B. (2003) *Mol. Cell. Neurosci.* **24,** 1103–1115
35. Malleret, G., Haditsch, U., Genoux, D., Jones, M. W., Bliss, T. V., Vanhoose, A. M., Weitlauf, C., Kandel, E. R., Winder, D. G., and Mansuy, I. M. (2001) *Cell* **104,** 675–686
36. Santana, L. F., Chase, E. G., Votaw, V. S., Nelson, M. T., and Greven, R. (2002) *J. Physiol.* **544,** 57–69
37. Takai, A., and Mieskes, G. (1991) *Biochem. J.* **275,** 233–239
38. Takai, A., Sasaki, K., Nagai, H., Mieskes, G., Isobe, M., Isono, K., and Yasumoto, T. (1995) *Biochem. J.* **306,** 657–665
39. Bialojan, C., and Takai, A. (1988) *Biochem. J.* **256,** 283–290
40. Marley, A. E., Sullivan, J. E., Carling, D., Abbott, W. M., Smith, G. J., Taylor, I. W., Carey, F., and Beri, R. K. (1996) *Biochem. J.* **320,** 801–806
41. Mukai, H., Kuno, T., Chang, C. D., Lane, B., Luly, J. R., and Tanaka, C. (1993) *J. Biochem.* (*Tokyo*) **113,** 292–298
42. Foor, F., Parent, S. A., Morin, N., Dahl, A. M., Ramadan, N., Chrebet, G., Bostian, K. A., and Nielsen, J. B. (1992) *Nature* **360,** 682–684
43. Rogers, J. P., Beuscher, A. E. T., Flajolet, M., McAvoy, T., Nairn, A. C., Olson, A. J., and Greengard, P. (2006) *J. Med. Chem.* **49,** 1658–1667
44. Sheppeck, J. E., 2nd, Gauss, C. M., and Chamberlin, A. R. (1997) *Bioorg. Med. Chem.* **5,** 1739–1750
45. Zhang, J., Ma, Y., Taylor, S. S., and Tsien, R. Y. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98,** 14997–15002
46. Zhang, J., Campbell, R. E., Ting, A. Y., and Tsien, R. Y. (2002) *Nat. Rev. Mol. Cell. Biol.* **3,** 906–918
47. Brennan, J. P., Bardswell, S. C., Burgoyne, J. R., Fuller, W., Schroder, E., Wait, R., Begum, S., Kentish, J. C., and Eaton, P. (2006) *J. Biol. Chem.* **281,** 21827–28136
48. Meinhard, M., and Grill, E. (2001) *FEBS Lett.* **508,** 443–446
49. Meinhard, M., Rodriguez, P. L., and Grill, E. (2002) *Planta* **214,** 775–782
50. Rhee, S. G., Bae, Y. S., Lee, S. R., and Kwon, J. (2000) *Sci. STKE 2000*, PE1
51. Ullrich, V., Namgaladze, D., and Frein, D. (2003) *Toxicol. Lett.* **139,** 107–110
52. Kim, H. S., Song, M. C., Kwak, I. H., Park, T. J., and Lim, I. K. (2003) *J. Biol. Chem.* **278,** 37497–37510
53. Wang, X., Culotta, V. C., and Klee, C. B. (1996) *Nature* **383,** 434–437
54. Bogumil, R., Namgaladze, D., Schaarschmidt, D., Schmachtel, T., Hellstern, S., Mutzel, R., and Ullrich, V. (2000) *Eur. J. Biochem.* **267,** 1407–1415
55. Fetrow, J. S., Siew, N., and Skolnick, J. (1999) *FASEB J.* **13,** 1866–1874
56. Hensley, K., and Floyd, R. A. (2005) *Antioxid. Redox Signal.* **7,** 523–525
57. Rhee, S. G., Chang, T. S., Bae, Y. S., Lee, S. R., and Kang, S. W. (2003) *J. Am. Soc. Nephrol.* **14,** S211–S215