Oxidant-induced Activation of Type I Protein Kinase A Is Mediated by RI Subunit Interprotein Disulfide Bond Formation*

Received for publication, April 25, 2006, and in revised form, June 2, 2006 Published, JBC Papers in Press, June 5, 2006, DOI 10.1074/jbc.M603952200

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Here we demonstrate that type I protein kinase A is redox-active, forming an interprotein disulfide bond between its two regulatory RI subunits in response to cellular hydrogen peroxide. This oxidative disulfide formation causes a subcellular translocation and activation of the kinase, resulting in phosphorylation of established substrate proteins. The translocation is mediated at least in part by the oxidized form of the kinase having an enhanced affinity for α-myosin heavy chain, which serves as a protein kinase A (PKA) anchor protein and localizes the PKA to its myofilament substrates troponin I and myosin binding protein C. The functional consequence of these events in cardiac myocytes is that hydrogen peroxide increases contractility independently of β-adrenergic stimulation and elevations of cAMP. The oxidant-induced phosphorylation of substrate proteins and increased contractility is blocked by the kinase inhibitor H89, indicating that these events involve PKA activation. In essence, type I PKA contains protein thiols that operate as reoxid sensors, and their oxidation by hydrogen peroxide directly activates the kinase.

There is now substantial evidence that oxidant species such as H₂O₂ are produced in a regulated way in cells where they can function as signaling agents (1, 2). We have been studying the post-translational modification of protein cysteinyi thiols, as this is a major mechanism by which oxidants can alter the structure of proteins and so regulate their function. Our strategy has been to search for proteins that are susceptible to a variety of different modes of cysteine oxidation, such as S-thiolation (3, 4), sulfenation (5), and protein-protein disulfide bond formation (6). The rationale is that once we identify proteins with reactive thiols, the possibility that their oxidation has a functional correlate of physiological significance can be investigated. We previously found the RI regulatory subunits of protein kinase A (PKA) form interprotein disulfide dimers during cardiac oxidative stress (6).

Here we investigated the potential impact of this disulfide dimer formation on the function of PKA. PKA has two major forms (type I and type II), both of which exist as a tetramer comprising two catalytic and two regulatory subunits. There are two types of regulatory subunits (RI and RII), the presence of which in the PKA holokinase nominally defines the enzyme as type I or II, respectively. Recent studies have shown that the full dissociation of type I PKA in response to cAMP requires the presence of a substrate (7). This substrate-induced sensitization of type I PKA is not a feature of the type II enzyme (8). The regulatory subunits contain N-terminal sequences that are important for protein kinase A anchor protein (AKAP) binding. AKAPs are a diverse group of proteins that are found next to PKA substrate proteins and, thus, function to target PKA (9). Type I PKA is located in the cytosol, whereas type II is not as a result of being primarily bound (targeted) to AKAP proteins that are associated with various subcellular compartments, including the myofilaments in myocytes.

RI and RII have significant homology, but one notable difference is the presence of a pair of N-terminal cysteine residues in RI. These cysteines have been thought to form constitutively present interprotein disulfides between RI subunits (10), which align anti-parallel to each other and form disulfide bonds linking Cys-17 and -38 (in rat) of different RI molecules (11). However, here we report RI does not, in fact, exist constitutively as a disulfide dimer. The RI disulfide dimer only forms when pro-oxidizing conditions exist. In cardiac tissue this redox change induces a subcellular translocation and kinase activation, resulting in phosphorylation of multiple PKA substrates, which increases the amplitude of myocyte contraction. The disulfide formation and PKA activation are causatively linked, as a selective inhibitor of this kinase (H89) prevents these changes. This oxidant-induced modification and activation of PKA as well as phosphorylation of established PKA substrates occurs without elevations in cAMP, further supporting a functional consequence of the oxidative structural modification.

* This research was supported by grants from The Wellcome Trust, the United Kingdom Biotechnology and Biological Sciences Research Council, and The British Heart Foundation. 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.

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2 The abbreviations used are: PKA, protein kinase A; AKAP, protein kinase A anchor protein; ARVM, adult rat ventricular myocytes; PBS-T, phosphate-buffered saline plus 1% Triton X-100; α-MyHC, α-myosin heavy chain.
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EXPERIMENTAL PROCEDURES

Animals—Animals received humane care in compliance with the “Principles of Laboratory Animal Care” as published by the National Institutes of Health (NIH Publication no. 85-23). Rats were anesthetized with sodium pentobarbitone (100 mg kg⁻¹ intraperitoneal).

Fresh and Cultured Rat Myocyte Preparation—Calcium-tolerant adult rat ventricular myocytes (ARVM) were isolated from male Wistar rats (250–300 g) as before (12) and kept at room temperature in modified Tyrode buffer for 3 h or cultured overnight as described previously (13). Fresh ARVM were treated with H₂O₂ (1 μM–10 mM) (Sigma) for 5 min. Cells were centrifuged at 500 × g for 30s and then reconstituted in non-reducing SDS sample buffer containing maleimide (100 μM). In a separate experiments ARVM were treated with H₂O₂ (100 μM) or isoprenaline (100 nM) (Sigma) for 5 min with or without a 5-min pretreatment of H89 (10 μM) (Calbiochem) and again prepared in maleimide sample buffer. In cultured ARVM, after 18 h of incubation H₂O₂ (1–1000 μM) was added to the culture medium for 5 min. The culture medium was then aspirated, and the myocytes were scraped into 100 μl of maleimide sample buffer.

Crystallloid Perfusion of the Rat Heart—Hearts from male Wistar rats were prepared and buffered-perfused as before (3). After 25 min of aerobic perfusion, hearts were perfused at a constant flow with Krebs-Henseleit bicarbonate buffer containing H₂O₂ (1 μM–10 mM) or isoprenaline (100 nM) for 5 min or with H₂O₂ (100 μM) for 1–10 min. Ventricles were snap-frozen and homogenized (10 ml buffer/g tissue) on ice in 1% Triton X-100 and centrifuged as above with maleimide buffer and diluted 10-fold with phosphate-buffered saline plus 1% Triton X-100 (PBS-T), supplemented with 0.1 ml of Triton X-100 (10% v/v), vortexed, and centrifuged at 21,000 × g for 5 min. A soluble fraction was separated on a 24 ml of Superose 12 column (Amersham Biosciences) using a Bio-Rad chromatograph collecting 0.75-ml fractions. Fractions were reconstituted in SDS sample buffer and analyzed for the distribution of the catalytic unit of PKA by immunoblotting.

cAMP-Agarose Affinity Capture and Protein Band Identifications—1 ml of Triton-soluble heart extract was prepared as above with maleimide buffer and diluted 10-fold with phosphate-buffered saline plus 1% Triton X-100 (PBS-T), supplemented with protease inhibitors, and rotated overnight at 4 °C with 100 μl of cAMP-agarose (A-7396, Sigma). The affinity matrix was spin-washed 5 times with 10 ml of PBS-T and reconstituted in non-reducing SDS sample buffer and resolved by SDS-PAGE. Unknown protein bands were identified as described previously (6, 14) by analysis of tryptic digests using a quadrupole-time of flight hybrid quadrupole/orthogonal acceleration time of flight spectrometer (Micromass, Manchester, UK) interfaced to a Micromass CapLC capillary chromatograph.

Immunoprecipitation—0.5 ml of Triton-soluble extracts were prepared as above and diluted 10-fold with PBS-T and additional protease inhibitors. This was rotated for 5 h at 4 °C with 5 μg of antibody to α-myosin heavy chain (α-MyHC) before rotation at 4 °C for 2 h with of 50 μl of protein G-Sepharose (Amersham Biosciences). After spin-washing (4X with PBS-T) the beads were reconstituted in SDS sample buffer and analyzed for the presence of α-MyHC and PKA by immunoblotting.

PKA-Myoflament/Membrane Binding Assays—Ventricular tissue from anaerobically perfused hearts was homogenized as above. The myofilaments and membranes from a 1-ml aliquot were pelleted by centrifugation (21,000 × g for 5 min) and spin-washed twice in PBS by resuspension and repelleting. The pellet was resuspended into 1 ml of PBS with protease inhibitors, and then 0.2-ml aliquots of this suspension was mixed with 0.8 ml of PBS containing 1 mg of type I PKA holoenzyme (Biafin, Kassel, Germany) with or without 100 mM dithiothreitol. The RI subunit of the kinase was supplied in the oxidized interprotein disulfide state, and 100 mM treatment efficiently reduced it to the free thiol state. After rotation at 4 °C for 2 h, the myofilaments and membrane fraction were repelleted, and the soluble fraction was reconstituted in SDS sample buffer. The pellet was then resuspended in PBS-T and centrifuged at 21,000 × g for 5 min to pellet the myofilaments but leave the Triton-soluble membranes in the supernatant. Both of these fractions were mixed with SDS sample buffer, and the relative distribution of Tyrode) for 5 min at room temperature. Cells were pelleted at 12,000 × g for 5 min, and the supernatant was discarded. 100 μl of “designer assay solution” was added to the pellet and vortexed. 90 μl of each sample was transferred to a 96-well black polypropylene microplate, covered, and incubated at room temperature for 30 min. The fluorescence intensity was read with a SpectraMax Gemini XPS fluorescence plate reader (Molecular Devices, Wokingham, UK) (excitation ~485 nm; emission ~530 nm).

Gel Filtration—Ventricular tissue was homogenized as described above but without maleimide. 0.9 ml of the homogenate was supplemented with 0.1 ml of Triton X-100 (10% v/v), vortexed, and centrifuged at 21,000 × g for 5 min. A soluble fraction was separated on a 24 ml of Superose 12 column (Amersham Biosciences) using a Bio-Rad chromatograph collecting 0.75-ml fractions. Fractions were reconstituted in SDS sample buffer and analyzed for the distribution of the catalytic unit of PKA by immunoblotting.

Antibodies Used in This Work—Primary antibodies used in these studies included PKA RI subunit (Calbiochem and Santa Cruz, Heidelberg, Germany), PKA-catalytic subunit (Transduction Laboratories, Oxford, UK), phospho-(Ser/Thr) PKA substrate (New England Biolabs, Hitchin, UK), phospho-phospholamban (Badrilla, Leeds, UK), phospho-protonin I (New England Biolabs, Hitchin, UK), and α-MyHC (Santa Cruz). Horseradish peroxidase-linked secondary antibody and ECL reagent (both Amsham Biosciences) were used to visualize the proteins.

cAMP Assay—cAMP was measured in cells treated with H₂O₂ or isoprenaline using a Bridge-It cAMP designer fluorescence assay kit according to the manufacturer’s guidelines (Mediomics). Myocyte aliquots were treated with H₂O₂ (1–1000 μM), isoprenaline (1–1000 nM), or control (modified
the added recombinant PKA was determined using an RI subunit antibody (Santa Cruz).

**Myocyte Contractility**—ARVM were placed in a chamber mounted on an inverted microscope (Nikon Eclipse) and visualized using a 40× objective. Sarcomere length was calculated in real time at 240 Hz by a video analysis system (IonOptix, Milton, MA). Cells were stimulated at 0.5 Hz and superfused with HEPES-based solution (120 mM NaCl, 5.4 mM KCl, 5 mM HEPES, 1 mM Na₂HPO₄, 1 mM MgSO₄, 10 mM glucose, 10 mM sodium acetate, and 1 mM CaCl₂) at 34 °C. After a stabilization period (>5 min), the response to continuous superfusion of 100 μM H₂O₂ was recorded in the presence and absence of H89 (1 μM). H89 was used at this concentration, as preliminary experiments demonstrated that 10 μM H89 decreased contractility of myocytes under control conditions, probably due to inhibition of basal PKA activity. An average of 10 twitches was used for each sample point.

**Immunofluorescence and Confocal Microscopic Imaging**—ARVM were cultured and prepared for immunofluorescence studies essentially as described previously (15). Briefly, ARVM were cultured overnight on sterile laminin-coated glass coverslips and exposed to H₂O₂ (100 μM) or modified Tyrode for 5 min. The myocytes were washed with PBS and fixed with 4% paraformaldehyde, PBS for 10 min. After washing cells were permeabilized with 0.1% Triton X-100, PBS for 20 min. ARVM were again washed and blocked with 1% bovine serum albumin, PBS for 60 min. After removal of blocking buffer, ARVM were then incubated with 1% bovine serum albumin, PBS containing PKA RI antibody (1:100) to detect cellular PKA RI. ARVM were then washed twice, and the coverslips were mounted on slides with fluorescent mounting medium (Dako Cytomation, Denmark). The slides were viewed on an inverted laser scanning microscope (LSM510, Carl Zeiss Inc) equipped with a 40×/1.3 NA Plan-Neofluar oil immersion objective lens (Carl Zeiss, Inc.), and images were acquired and processed using LSM510 software (Version 2.01).

**Statistical Analysis**—Differences between groups were assessed using analysis of variance with further analysis using Dunnett’s test or the Student-Newman-Keuls test (for multiple comparisons). Paired t tests were used to analyze amplitude data from myocyte contractility studies. p < 0.05 was considered significant. Data points shown are the means ± S.E.

**RESULTS**

**PKA RI Dimer Formation**—Fig. 1, a–c, shows that H₂O₂ treatment of freshly isolated or cultured ARVM as well as isolated rat hearts cause the RI subunit of PKA to form an SDS-
**FIGURE 2. PKA substrate phosphorylation after H$_2$O$_2$ treatment.** Panels a and b show phosphorylation of 10- and 25-kDa myocyte proteins in response to H$_2$O$_2$ (5 min), detected by immunoblotting using a pan-specific antibody to phosphorylated PKA substrates. Quantitative analysis ($n = 3$) confirmed that H$_2$O$_2$ treatment induced a progressive increase in phosphorylation of both these proteins, which from their molecular masses were considered likely to be phospholamban and troponin I, respectively. The bell-shaped phosphorylation response of both these proteins was maximal at 100 $\mu$M H$_2$O$_2$, after which there is a loss of phosphorylation. Panel c shows similar findings to those in a and b in cultured myocytes treated with 100 $\mu$M H$_2$O$_2$ (5 min), but these studies instead utilized individual phosphospecific antibodies to phospholamban and troponin I, confirming oxidant-induced phosphorylation of these proteins and corroborating the observations made with the pan-specific phospho-PKA substrate antibody. Panel d shows a Western blot probed with the phospho-PKA substrate antibody and shows that treatment of myocytes with H$_2$O$_2$ (100 $\mu$M, 5 min) or isoprenaline (100 nM, 5 min) caused phosphorylation of multiple substrates. Pretreatment of cells with the PKA inhibitor H89 (10 $\mu$M, 5 min) blocked the effect of H$_2$O$_2$ but was a less effective in attenuating the increases induced by a high dose of isoprenaline. It is notable that the pattern of phosphorylation by isoprenaline is virtually identical to that of H$_2$O$_2$, consistent with H$_2$O$_2$-induced PKA activation. IB, immunoblot. C, control.
resistant dimer. This dimer formation is reversed by 2-mercaptoethanol, indicating that the gel shift is due to an intermolecular disulfide bond (6). Disulfide formation is dose-dependent, with more than 60% of PKA RI in freshly isolated ARVM becoming dimeric after 5 min with 100 μM H₂O₂. Similar quantitative changes were also observed with cultured preparations and in isolated hearts (not shown). In the same samples we observed no changes in the migration of the PKA catalytic subunit (not shown).

PKA Substrate Phosphorylation—Using a pan-specific phospho-PKA substrate antibody (which recognizes RXRXL or RXRPS; pT and pS are phosphothreonine and phosphoserine, respectively), we observed phosphorylation of several PKA substrates after H₂O₂ treatment of myocytes. Fig. 2, a and b, shows a bell-shaped phosphorylation response by 10- and 25-kDa proteins present in ARVM in response to H₂O₂. The 10- and 25-kDa substrate proteins were suspected to be phospholemman and troponin I, which was confirmed using additional phospho-specific antibodies to these individual proteins (Fig. 2c). Fig. 2d also shows that H₂O₂ (100 μM) induced phosphorylation of phospholemman and troponin I, but it is clear that two other PKA substrates proteins are phosphorylated. These proteins are likely to be the PKA sarcolemmal substrate phospholemman (15 kDa) and the myofilament protein myosin-binding protein C (150 kDa), as these bands co-migrate with antibodies to these proteins (not shown). Fig. 2d also shows that the PKA inhibitor H89 (10 μM) blocked the H₂O₂-induced phosphorylation of PKA substrate proteins. As expected, isoprenaline caused robust activation of the same substrate proteins, although this was not fully H89-sensitive, probably as a result of the high agonist dose. In separate experiments (not shown) we used isoprenaline and H89 at lower concentrations. H89 (1 μM) still blocked H₂O₂-induced phosphorylation. We also observed a more pronounced H89-induced attenuation of substrate phosphorylation when PKA was stimulated with 10 nM isoprenaline.

**cAMP Concentration Does Not Change after H₂O₂ Treatment**—Treatment of ARVM with isoprenaline caused a 2.3-fold change in fluorescence due to increased cAMP concentration (see Fig. 3). In comparison, H₂O₂ treatment did not cause any alterations in fluorescence/cAMP in these cells.

**PKA RI Translocation**—Fig. 4a shows PKA RI subcellular distribution in the isolated heart after treatment with 100 μM H₂O₂ for 1–10 min. PKA RI is a cytosolic monomer that undergoes time-dependent dimerization during H₂O₂ treatment. The RI dimer translocates to the myofilament compartment, with some evidence on long Western blot exposures of a small increase in the membrane fraction also. This RI translocation was also seen in ARVM after H₂O₂ treatment without any translocation of PKA RI subunit (not shown). Isoprenaline treatment did not induce disulfide formation or subcellular translocation. This translocation was confirmed in immunofluorescence confocal imaging studies shown in Fig. 4b. There is a clear translocation of PKA RI to the nucleus of myocytes treated with H₂O₂. This increased nuclear localization was also accompanied by a less striking increase in myofibrillar localization as evidenced by an enhancement in the striated pattern of the H₂O₂-treated cells. However, although the nuclear translocation is very pronounced and robust, the enhanced filament staining is a more subjective interpretation and is a common issue encountered when interpreting immunofluorescence micrographs of adult ventricular myocytes. We examined this increased affinity of the RI disulfide dimer for the myofilament/nuclear fraction in *in vitro* reconstitution studies. Fig. 4c shows PKA RI in the oxidized disulfide, but not the reduced thiol, state has affinity with the myofilament/nuclear fraction. This demonstrates the importance of disulfide formation for subcellular relocalization during H₂O₂ treatment.

**Peroxide-induced Dissociation of PKA Holoenzyme**—We also assessed the size of the kinase molecular complex using gel filtration, as the holoenzyme dissociates during activation. Fig. 5 shows the size distribution of the complexes containing the catalytic unit of PKA in isolated hearts treated with H₂O₂, and also a control sample incubated with maleimide. Under basal conditions the holoenzyme exists as a four subunit complex, eluting predominantly in fractions 5–6. With increasing H₂O₂ concentrations the holoenzyme complex breaks down, with the catalytic subunit eluting in progressively later fractions. Maleimide also caused holoenzyme dissociation, perhaps providing evidence that thiol oxidation involving alkylation in addition to interprotein disulfide formation might activate the kinase.

Immunoblot analysis of the regulatory subunits in these samples was also undertaken. However, because these subunits remain as dimeric complexes regardless of oxidation state, the molecular mass only changes from ~200 to ~100 kDa during kinase activation and dissociation. The Superoxide 12 column is unable to resolve adequately over this range, and so, as expected, there is little variation in the distribution of the regulatory subunit between treatment groups (not shown).

**cAMP-agarose Affinity Capture and Co-immunoprecipitation Studies**—We investigated the possibility that formation of the PKA RI disulfide dimer leads to increased affinity with a substrate or associated binding protein, acting as a targeting event that co-localizes kinase and substrate. Such an event
would also account for the H$_2$O$_2$-mediated translocation. Consequently, we used cAMP-agarose to affinity-purify the regulatory subunit of PKA along with any proteins that are differentially associated after H$_2$O$_2$ treatment. Fig. 6a shows a Western blot of the affinity-purified preparations probed with a PKA RI antibody, demonstrating binding of both the reduced form and oxidized disulfide form of RI. Fig. 6b shows a Coomassie-stained gel of cAMP-agarose affinity preparations, demonstrating increasing amounts of a high molecular weight protein as the H$_2$O$_2$ dose increased. This protein was identified as $\alpha$-MyHC in four separate experiments and, as discussed below, highlights a novel role for this protein as an AKAP. In reciprocal experiments we immunoprecipitated $\alpha$-MyHC to further test its interaction with RI as it dimerizes (Fig. 6c). Again, we observed an increased association of $\alpha$-MyHC with RI in ARVM in a H$_2$O$_2$ dose-dependent manner. Similar results were also obtained with immunoprecipitations from isolated heart preparation (not shown).
following oxidative disulfide formation. The N terminus of the RI molecule where the dimer interaction occurs contains the cysteine residues (Cys-17 and -38 in rat) that form disulfides. This N-terminal dimerization event is at the opposite end from the cAMP binding sites, which are involved in holoenzyme dissociation and activation. Our studies with cAMP-agarose (Fig. 6) show that both the reduced and oxidized disulfide forms of the RI subunit bind cAMP.

The oxidative disulfide formation in RI is associated with subcellular translocation of type I PKA from the cytosol to the nuclear and myofilament compartment and to a lesser extent, a membrane fraction as demonstrated by immunoblotting of subcellular fractions of heart tissue and immunofluorescence confocal imaging of intact myocytes (Fig. 4). In this connection it is of note that Boeshans et al. (16) demonstrated that a primary difference between bovine cardiac cytosolic RI and that associated with the membrane was that the latter “had a higher extent of disulfide bond formation in the N-terminal dimerization domain,” although they concluded, “This was not likely the cause for membrane localization.” However, our observations suggest that the presence of the disulfide is important for subcellular localization. This redistribution is accompanied by phosphorylation of established PKA substrate proteins in these fractions, including troponin I, myosin-binding protein C, and phospholamban. These phosphorylation events are sensitive to the inhibitor H89, demonstrating phosphorylation is likely mediated by PKA. Other kinases will likely be activated by peroxide, but these would have to also phosphorylate established PKA substrates and at the same time be sensitive to H89. It is also notable that the profile of phosphorylation detected using the pan-specific PKA-substrate antibody is very similar regardless of H2O2 or isoprorenaline treatment, although the latter intervention gave stronger signals, perhaps explained by simultaneous activation of type II PKA. It is possible that future mutagenesis studies involving the replacement of either or both of the redox-active cysteines in RI would help to unequivocally link disulfide formation to kinase activation. However, how such mutants will behave is difficult to anticipate with certainty. It remains possible that amino acid substitutions could alter the RI dimer interaction, possibly constitutively activating the kinase if the RI interaction was fully disrupted. Also, because this is the AKAP binding domain, cysteine replacement mutants may have altered interactions with these targeting proteins, adding further complexity to the study of oxidant-induced activation involving disulfide formation.

**DISCUSSION**

Here we demonstrate that the regulatory subunit of type I PKA has cysteinyl thiols that sense the prevailing cellular redox status. Elevated cellular H2O2 causes the two RI subunits of the tetrameric holoenzyme to form interprotein disulfides. This disulfide was already known but to date was considered a constitutive structural bond (10, 11). RI has probably been purified as a dimer because of its oxidant sensitivity, rapidly forming a disulfide bond on exposure to air during preparation.

We previously observed that PKA RI exists in the non-dissulfide state in control tissue, only forming the disulfide dimer in response to pro-oxidants (6). We could make these observations as our samples were prepared with maleimide, which alkylates the thiol and prevents subsequent oxidative 
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One possibility was that the H2O2 treatment somehow activated adenylyl cyclase to elevate cAMP and activate PKA, as with β-stimulation. However, there was no elevation in cAMP after H2O2 treatment (Fig. 3), consistent with studies demonstrating inhibition of adenylyl cyclase by oxidants (17, 18). So disulfide formation activates type I PKA without an elevation in cAMP, and this is associated with subcellular relocalization to compartments containing PKA substrates. To obtain another index of the activity status of PKA after H2O2 intervention, we assayed the molecular complex size of the enzyme by gel filtration chromatography. Our interpretation of these results is that under basal conditions the catalytic subunit is part of an intact tetrameric complex (containing two catalytic and two regulatory subunits) and, therefore, elutes in early fractions. After H2O2 treatment the catalytic subunit is released from the complex and is free to elute in its monomeric state in later fractions. The decrease in PKA complex size, highlighting the dissociation of the catalytic subunit from the holoenzyme complex, provided additional evidence of kinase activation after H2O2 treatment.

We can explain these observations regarding oxidant-induced activation of this kinase in light of recent studies demonstrating that type I PKA, in contrast to type II, is activated by substrate-induced sensitization to cAMP (7, 8). Also, type I is generally accepted to activate at lower cAMP concentration than type II. Thus, the translocation of type I PKA during oxidative stress is crucially important for its activation, because it brings the kinase close to its physiological substrates. We reasoned disulfide formation in type I PKA must increase the affinity of the kinase with its substrates or with an associated protein that enables localization with substrate. AKAPs are a diverse array of proteins that allow molecular targeting of PKA to its multiple and variously distributed substrates (9). Type I PKA is generally cytosolic and not constitutively associated with AKAP proteins, unlike the type II isoform that is targeted to AKAP-substrate complexes. To investigate the possibility that the redox status of type I PKA regulates its affinity with substrate complexes, we compared the ability of type I PKA in the reduced or disulfide state to bind cardiac myofilament/membrane preparations. These studies verified that the oxidized, but not the reduced, form of type I PKA binds to the myofilament compartment (Fig. 4c). This demonstrates that disulfide formation in the RI subunit operates as a redox switch controlling distribution in both this in vitro binding assay and in cells. To further understand this targeting event, we undertook affinity purifications of the regulatory subunit of PKA from control- or H2O2-treated preparations using cAMP-agarose, which we knew bound reduced and oxidized disulfide forms of RI. We looked for proteins unique to preparations from tissue treated with H2O2, as any such proteins might represent an AKAP that binds the disulfide form of RI to effect subcellular translocation. This was considered a possibility because the regulatory subunits of PKA contain AKAP binding sequences, which are in proximity to the redox active cysteines.

These cAMP-agarose binding studies identified α-MyHC as a protein that behaved in this way. Although this protein is not an established AKAP, our identification of it interacting with PKA would highlight this function. α-MyHC has a well defined role in cardiac muscle contractility, but its function as an AKAP is rational as it would localize PKA close to its myofilament substrates troponin I and myosin-binding protein C. Indeed, these proteins were phosphorylated after H2O2 treatment (Fig. 2) at the same time as the RI disulfide dimer forms and translocates to the myofilaments by, probably, α-MyHC. Recent studies have demonstrated that several myosins function as...
AKAPs (19, 20). The rod domain of MyHC contains an amphipathic α-helix stabilized in a coiled-coil configuration. This helix could function like amphipathic helices found in AKAPS and serve to localize PKA, although in this case it would be dependent on RI being in an oxidized disulfide state. Further evidence for α-MyHC functioning as an AKAP that localizes oxidized PKA comes from immunoprecipitation studies showing that RI co-purified with α-MyHC progressively more as the dose of H$_2$O$_2$ was increased in cells (Fig. 6). It is also notable that α-MyHC has been identified as a protein kinase G-anchoring protein (21), providing further support for this interaction as a targeting mechanism.

The increase in contractility in response to β-adrenergic stimulation is mediated by cAMP-dependent phosphorylation of substrates by PKA, including those proteins we showed were phosphorylated after H$_2$O$_2$ treatment. Consequently, one of the predicted consequences of H$_2$O$_2$ treatment would, therefore, be increased myocyte contractility, which was what we observed. This contrasts other work demonstrating negative inotropic responses with H$_2$O$_2$ treatment (22, 23), although in some studies initial increases in contractility were observed (24). However, many of these studies showing H$_2$O$_2$-induced loss of contractility were carried with higher concentrations or for extended treatment times. Furthermore, these studies had the underlying hypothesis that oxidants adversely affect cardiac function, perhaps leading to the use of H$_2$O$_2$ at concentrations that produce the anticipated detrimental effects. However, in one study, using lower concentrations similar to those used here, H$_2$O$_2$ also substantially enhanced contractility (25). Although we were not able to measure phosphorylation or activation of the L-type calcium channel in these studies, previous work demonstrated its activation in cardiac myocytes after H$_2$O$_2$ treatment (26). This would lead to increased intracellular calcium that would further contribute to the inotropic effect of H$_2$O$_2$ treatment.

The catalytic subunit of PKA is also susceptible to cysteine oxidation and is inhibited by S-thiolation, which is enhanced when the kinase is activated (27, 28). We have not assessed catalytic subunit S-thiolation here, but we did not detect any shift in the migration of the catalytic subunit in any of our H$_2$O$_2$-treated samples. This is in contrast to the observations of Humphries et al. (27), who found oxidative stress was associated with both interprotein disulfide bond formation (large gel shift to a higher mass) as well as intramolecular disulfide formation, which caused a faster migrating species and a small shift down the gel. We have also previously screened for proteins susceptible to cysteine-targeted oxidation including S-thiolation (3–6, 14) and found no evidence of catalytic subunit modification, although we could have missed it due to low abundance. However, it is clear from the studies here that in cardiac cells and tissues exposure to H$_2$O$_2$ activates type I PKA.

Our novel observations may help shed some light on the tel-
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...ological purpose of two isoforms of PKA. It is possible that type I PKA activity is primarily coupled to the cellular redox state and is activated by H$_2$O$_2$, whereas the role of type II is the classical activation in response to cAMP via β-adrenergic stimulation. This work also highlights how type I PKA, which is predominantly cytosolic under basal conditions, is able to phosphorylate its substrates located elsewhere in the cell. Activation of PKA independently of increases in cAMP has important implications for our view of β-receptor signaling. Clearly, many cellular events can lead to increases in cellular oxidants such as H$_2$O$_2$ (1, 2). The oxidant load of cells not only increases during times of increased metabolic activity but via many neurohormonal pathways that couple to phosphorylation-dependent oxidase activation (29, 30). As highlighted in Fig. 8, this oxidant-induced activation of PKA via RI interprotein disulfide formation provides a mechanism whereby the redox status of cells can integrate into control mechanisms involving phospho-regulation. Many disease states are characterized by oxidative stress, and one consequence of our observations is that such tissues might have abnormal or chronic stimulation of type I PKA, a possibility that warrants further attention.

Acknowledgments—We thank Professors Avkiran, Marber, and Shattock for helpful discussions.

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