The activity of cGMP-dependent protein kinase Iα is not directly regulated by oxidation-induced disulfide formation at cysteine 43

Hema Kalyanaraman, Shunhui Zhuang, Renate B. Pilz, and Darren E. Casteel

From the Department of Medicine, University of California, San Diego, La Jolla, California 92093

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The type I cGMP-dependent protein kinases (PKGs) are key regulators of smooth muscle tone, cardiac hypertrophy, and other physiological processes. The two isoforms PKGⅠα and PKGⅠβ are thought to have unique functions because of their tissue-specific expression, different cGMP affinities, and isoform-specific protein-protein interactions. Recently, a non-canonical pathway of PKGⅠα activation has been proposed, in which PKGⅠα is activated in a cGMP-independent fashion via oxidation of Cys43, resulting in disulfide formation within the PKGⅠα N-terminal dimerization domain. A “redox-dead” knock-in mouse containing a C43S mutation exhibits phenotypes consistent with decreased PKGⅠα signaling, but the detailed mechanism of oxidation-induced PKGⅠα activation is unknown. Therefore, we examined oxidation-induced activation of PKGⅠα, and in contrast to previous findings, we observed that disulfide formation at Cys43 does not directly activate PKGⅠα in vitro or in intact cells. In transfected cells, phosphorylation of Ras homolog gene family member A (RhoA) and vasodilator-stimulated phosphoprotein was increased in response to 8-CPT-cGMP treatment, but not when disulfide formation in PKGⅠα was induced by H2O2. Using purified enzymes, we found that the Cys43 oxidation had no effect on basal kinase activity or $K_m$ and $V_{max}$ values; however, PKGⅠα containing the C43S mutation was less responsive to cGMP-induced activation. This reduction in cGMP affinity may in part explain the PKGⅠα loss-of-function phenotype of the C43S knock-in mouse. In conclusion, disulfide formation at Cys43 does not directly activate PKGⅠα, and the C43S-mutant PKGⅠα has a higher $K_m$ for cGMP. Our results highlight that mutant enzymes should be carefully biochemically characterized before making in vivo inferences.

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To whom correspondence should be addressed: Dept. of Medicine, University of California, San Diego, 9500 Gilman Dr., La Jolla, CA 92093-0652. Tel.: 858-534-8806; Fax: 858-534-1421; E-mail: dcasteel@ucsd.edu.

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end of the PKGα LZ, activated the kinase in a cGMP-independent manner. Although PKG dimer formation is stably mediated by the LZ, Cys\textsuperscript{43} oxidation and disulfide formation cause the two peptide chains in the dimer to become covalently linked. In addition to direct kinase activation, Cys\textsuperscript{43} oxidation has been proposed to alter the $K_m$ and $V_{max}$ for substrates and to increase PKGα binding to specific interacting proteins (12).

A knock-in mouse containing a “redox-dead” C43S-mutant PKGα shows phenotypes consistent with a loss of PKGα signaling (13–19), and these results have been used to argue for a predominant role for Cys\textsuperscript{43} oxidation in PKGα regulation. However, the enzymatic properties of the C43S mutant PKGα were not thoroughly investigated.

Exactly how Cys\textsuperscript{43} oxidation activates PKGα is unknown. To explore the underlying mechanism, we began by comparing the activity of wild-type and C43S-mutant PKGα under reducing and oxidizing conditions. Surprisingly, and in contrast to previous findings, we found that wild-type PKGα activity was not directly increased by disulfide formation at Cys\textsuperscript{43} and that Cys\textsuperscript{43} oxidation had no effect on substrate phosphorylation. In addition, we found that the C43S mutation caused PKGα to have a lower sensitivity to cGMP-induced activation.

**Results**

**Oxidation-induced PKGα dimerization does not lead to increased kinase activity in intact cells**

In the original report describing activation of PKGα by Cys\textsuperscript{43} oxidation, A10 cells were transfected with wild-type or C43S-mutant PKGα and treated with $\text{H}_2\text{O}_2$, and myosin light chain phosphorylation was examined (12). $\text{H}_2\text{O}_2$ treatment caused increased PKGα disulfide bond formation in wild-type but not C43S-mutant PKGα, and disulfide bond formation was correlated with decreased myosin light chain phosphorylation, presumably through PKGα-induced activation of myosin phosphatase (5). Using a similar rationale, we examined whether PKGα Cys\textsuperscript{43} oxidation led to increased phosphorylation of known PKGα substrates in cells. We transfected 293T cells with expression constructs for RhoA together with wild-type or C43S-mutant PKGα and treated cells with 8-CPT-cGMP or $\text{H}_2\text{O}_2$. 8-CPT-cGMP-treated cells showed robust RhoA phosphorylation with both wild-type and C43S-mutant PKGα (Fig. 1A, top panel, compare lanes 1 and 2 and compare lane 4 and 5). As expected, treatment with $\text{H}_2\text{O}_2$ caused a pronounced increase in disulfide bond formation in wild-type but not C43S-mutant PKGα (shown by gel shift under non-reducing conditions; Fig. 1A, bottom panel, compare lanes 1 and 3 and compare lanes 4 and 6). Unexpectedly, $\text{H}_2\text{O}_2$ treatment had no effect on RhoA phosphorylation (Fig. 1A, top panel, compare lanes 1 and 3). Similar results were seen when vasodilator-stimulated phosphoprotein (VASP) phosphorylation was examined. PKG phosphorylates VASP at Ser\textsuperscript{157} and Ser\textsuperscript{239}, and phosphorylation at Ser\textsuperscript{157} causes VASP to migrate with a higher apparent molecular weight. Upon Western blotting of transfected cells probed with a VASP Ser\textsuperscript{P}\textsuperscript{239} antibody, VASP runs as a doublet, and 8-CPT-cGMP treatment causes VASP to completely shift to the upper band, indicating double phosphorylation of Ser\textsuperscript{157} and Ser\textsuperscript{239} (Fig. 1B, compare lanes 1 and 2 and compare lanes 4 and 5). However, in $\text{H}_2\text{O}_2$-treated cells, there is only a slight shift in VASP migration, and this shift is seen in cells transfected with wild-type or C43S-mutant PKGα, indicating that it is not due to Cys\textsuperscript{43} oxidation (Fig. 1B, compare lanes 1 and 3 and lanes 4 and 6). These results strongly suggest that Cys\textsuperscript{43} disulfide bond formation does not activate PKGα in cells.

**Oxidation of PKGα Cys\textsuperscript{42} does not increase kinase activity in vitro**

To directly examine whether disulfide formation at Cys\textsuperscript{43} increases kinase activity, we performed in vitro kinase assays. We used anti-Flag affinity beads to purify Flag-tagged PKGα from transiently transfected 293T cells; this one-step purification protocol allowed us to quickly isolate highly purified full-length wild-type and C43S-mutant PKGα (Fig. 2A). The purified kinases were incubated for 1 h in 15 mM DTT or allowed to oxidize by exposure to air in the absence of DTT. Immediately prior to measuring kinase activity, aliquots were removed for analysis by non-reducing SDS-PAGE/immunoblotting (as seen in Fig. 2B); under these conditions wild-type PKGα was $\sim 3.8\%$ oxidized in the presence of DTT and $\sim 59.7\%$ oxidized in the absence of reducing agent. As expected, C43S-mutant PKGα did not form a disulfide bond and ran as a monomer under both

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**Figure 1. PKGα is not activated in intact cells by disulfide formation at Cys\textsuperscript{43}.** A, 293T cells were transfected with expression vectors for Flag-tagged RhoA (500 ng) and untagged wild-type or C43S-mutant PKGα (500 ng). The cells were treated for 60 min with 250 μM 8-CPT-cGMP, 100 μM $\text{H}_2\text{O}_2$, or vehicle alone, as indicated. RhoA Ser\textsuperscript{188} phosphorylation was determined by SDS-PAGE under non-reducing conditions followed by immunoblotting with a RhoA Ser\textsuperscript{P}\textsuperscript{188} specific antibody. The amount of monomeric reduced (R) and disulfide-linked oxidized (O) PKGα was determined by blotting with a PKGα specific antibody. B, performed as in A, except 293T cells were transfected with expression vectors for VSV-tagged VASP (200 ng) and wild-type or C43S-mutant PKGα (200 ng), and blots were probed for VASP Ser\textsuperscript{P}\textsuperscript{239}. These experiments were repeated at least three times with similar results.

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**Figure 2. PKGα is not activated in vitro by Cys\textsuperscript{43} disulfide bond formation.**
PKG\(\alpha\) is not activated by \(\text{Cys}^{43}\) disulfide bond formation

Figure 2. Cys\(^{43}\) oxidation does not activate PKG\(\alpha\) in vitro. A, Coomassie-stained gel demonstrating the purity and integrity of Flag-tagged wild-type and C43S-mutant PKG\(\alpha\) isolated from transiently transfected 293T cells. B, purified wild-type and C43S-mutant PKG\(\alpha\) were incubated in the presence or absence of 15 mM DTT and exposed to air for 1 h; the amount of Cys43-cross-linked PKG\(\alpha\) enzyme kinetics (12). In previous studies, Burgoyne et al. (12) showed unique features that play important roles in regulating cGMP-induced kinase activity, including the structural basis for cGMP selectivity and novel interchain contacts that regulate kinase activation (10, 20, 21). Another recently proposed mechanism for PKG\(\alpha\) regulation was direct activation by oxidation-induced disulfide bond formation at Cys\(^{43}\). How the formation of a disulfide bond at Cys\(^{43}\) activates the kinase was not determined, and investigating the mechanism was the starting point for our current study.

In contrast to previous reports, we found that oxidation of PKG\(\alpha\) at Cys\(^{43}\) does not directly activate PKG\(\alpha\). In addition, Cys\(^{43}\) oxidation had no effect on substrate affinity or reaction velocity using Glassstide as a substrate, and oxidation did not lead to increased histone H1 phosphorylation. Although it is unclear why our results differ from earlier studies, one factor
may be that the original studies were performed using PKG\textsubscript{I\alpha} purchased from a commercial vendor and that the kinase could only be stimulated 1.5–3-fold by cGMP (12). This suggests that the kinase was proteolytically degraded, leading to a largely cGMP-independent kinase activity, and the poor quality of the enzyme may have affected the biochemical assays. In a subsequent paper by Prysyazhna et al. (16), PKG\textsubscript{I\alpha} was purified using a cAMP-agarose affinity column, followed by cAMP elution and dialysis to remove cAMP. This kinase could be stimulated 8-fold by cGMP as determined by Western blotting performed with phospho-specific antibodies to detect RhoA and histone H1 phosphorylation (see Fig. 6A in Ref. 16). In our experience, it is very difficult to fully remove cAMP during PKG purification, especially from PKG\textsubscript{I\alpha} (10, 20, 22), and in general, Western blotting is semi-quantitative and is not an accurate method to measure kinase activity.

It could be argued that the N-terminal Flag tag on our PKG\textsubscript{I\alpha} constructs affected kinase activity; however, we note that the kinase used in the present studies was purified intact, had a low basal activity, and could be stimulated 20–50-fold by cGMP. In addition, the Flag-tagged wild-type kinase had a $K_a$ for cGMP of 72–74 M, which is consistent with previous results measuring the $K_a$ of untagged PKG\textsubscript{I\alpha} in cell lysates (23, 24). Our $V_{\text{max}}$ and $K_m$ values for Glasstide differ from those obtained by Glass and Krebs ($K_m = 28.8 \text{ M}$ and $V_{\text{max}} = 20 \text{ pmol/min/\mu g}$ (25)); the different values may reflect the variation in kinase assay conditions, because their assays were performed using 2 mM Mg\textsuperscript{2+}, whereas we used 10 mM Mg\textsuperscript{2+}. In addition, Glass and Krebs had found that for small peptide substrates, PKG\textsubscript{I\alpha} activity peaked at 2 mM Mg\textsuperscript{2+} and rapidly decreased as free Mg\textsuperscript{2+} levels increased, whereas for large substrates like histone, activity steadily increased as free Mg\textsuperscript{2+} reached 75 mM (26). In comparison with our assays, previous in vitro kinase assays determining
PKGα is not activated by Cys\textsuperscript{43} disulfide bond formation

the effects of Cys\textsuperscript{43} oxidation were performed with either 5 or 15 mM MgCl\textsubscript{2}\textsuperscript{+} (12, 16). We should also point out that the transfected kinases used for our in-cell assays were without epitope tag (Fig. 1), demonstrating that oxidation did not increase the activity of untagged PKGα in cells. Therefore, we do not feel that the N-terminal Flag tag affected our results. We found that C43S-mutant PKGα has an ∼5-fold lower affinity for cGMP compared with the wild-type enzyme. This is an important finding, because mice homozygous for C43S-mutant PKGα have phenotypes consistent with a loss of PKGα function (17–19). These phenotypes include hypertension, insensitivity to nitroglycerin-induced vasodilation, and protection from septic shock; they have been interpreted to be the result of defective redox-sensing properties normally ascribed to Cys\textsuperscript{43} disulfide bond formation. However, mice null for the β\textsubscript{1} subunit of soluble guanylate cyclase are also resistant to nitroglycerin-induced vasodilation (27), indicating that the canonical NO-cGMP-PKG pathway is important and that oxidation sensing by Cys\textsuperscript{43} disulfide formation is not the main mechanism for nitroglycerin-induced vasodilation. Although our current studies have not looked at PKGα oxidation in mice, our results strongly suggest that defective signaling in PKGα C43S mice is due at least in part to an increased \( K_{n} \) for cGMP rather than a loss of redox-induced activation of the enzyme.

It is possible that loss of Cys\textsuperscript{43} oxidation might also affect other aspects of PKGα signaling; for example, oxidation of PKGα may alter its interaction with specific interacting proteins, which have been shown to bind to the PKGα leucine zipper (5, 7, 28). Indeed, oxidation has been shown to increase the in vitro interaction between PKGα and two of its interacting proteins, RhoA and MYPT1 (12). In addition, a recent paper by Nakamura et al. (15) demonstrated that the C43S mutation appears to alter PKGα subcellular localization in cardiac myocytes, which suggests a change in association of with interacting/anchoring proteins in cells, but the structural basis for oxidation-induced changes in these interactions was not examined. We are currently pursuing these studies.

How does the C43S mutation lead to decreased cGMP affinity? Since PKGα and PKGβ were first purified, it has been known that their different N termini cause the two isofoms to have different \( K_{n} \) values for cGMP, even though the sequences of the cyclic-nucleotide-binding pockets are identical (23). We have previously used hydrogen/deuterium-exchange mass spectrometry to study the PKGβ regulatory domain and found that, in the presence of the N-terminal LZ and autoinhibitory subdomains, hydrogen/deuterium exchange was increased throughout the cGMP-binding pockets (29). The increased conformational dynamics correlate with increased cGMP affinity. Because small molecule ligands are thought to stabilize pre-existing protein conformations (30, 31), we reasoned that the N terminus shifted the ensemble of conformations that PKGβ adopts in solution, such that the cyclic nucleotide-binding pockets spend more time in conformations that resemble the cGMP-bound forms. Therefore, it is possible that the C43S mutation, which lies at the end of the LZ, causes a change in the conformational dynamics of the nucleotide-binding pockets, which leads to lower cGMP affinity. In a previous analysis of the PKGα LZ, the C43S mutation lowered the \( T_{m} \) for thermal denaturation from 108 to 81.4 °C under oxidizing conditions and from 93.0 to 83.3 °C under reducing conditions (32). Although these melting temperatures are not physiological, thermal denaturation measures melting of the entire LZ, and the lower \( T_{m} \) in the C43S-mutant samples may reflect structural destabilization of the region surrounding Cys\textsuperscript{43}, which could occur in the full-length protein under physiological conditions.

In conclusion, we found that disulfide formation at Cys\textsuperscript{43} does not directly activate PKGα in vitro or in intact cells. In addition, “redox dead” C43S-mutant PKGα has a higher \( K_{n} \) for cGMP, and this decreased cGMP affinity may at least partially explain the loss-of-function PKGα phenotype observed in the C43S knock-in mice. Our results also highlight the general fact that mutant enzymes should be carefully characterized biochemically before cellular or physiological inferences are made.

Experimental procedures

Antibodies and reagents

Antibodies specific for Ser(P)\textsuperscript{188} of RhoA (sc-32954; lot no. A0914) and PKGα/β (sc-25429; lot no. F0910) were from Santa Cruz Biotechnology. Anti-VASP Ser(P)\textsuperscript{239} antibody was from Cell Signaling Technology (31145; lot no. 5). Anti-Flag M2 antibody F1804; lot no. 101M6216), anti-Flag M2 affinity gel, and Flag peptide were from Sigma. Horseradish-peroxidase-conjugated goat anti-mouse (115-035-062) and goat anti-rabbit (111-035-046) antibodies were from Jackson ImmunoResearch. Kemptide and Glasstide were from AnaSpec, Inc. Histone H1 (sc-221729; lot no. J2115) was from Santa Cruz Biotechnology.
General laboratory reagents were from Fisher Scientific, Sigma Life Science, or Bio-Rad.

**DNA constructs**

Expression vectors for untagged PKG\(\alpha\), Flag-RhoA, and VSV-VASP have been described previously (33–35). The Flag-tagged expression vector pFlag-D was constructed by annealing the oligonucleotides 5'-AGCTGCCACATGGACTACAAGAAGTCTATATCCCATC-3' (sense) and 5'-GATCCCTTGTCGTCATCGTCTTTGTAGTCCATGGTGG-3' (antisense) and ligating them into HindIII/BamHI-cut pcDNA3. Flag-tagged PKG\(\alpha\) was generated by PCR using the following set of primers: 5'-GACGGATCCGCCGCCATGAGCTAGCTAGAAGGAAG-3' (sense) and 5'-GCACCTCGAGTTATAGTCTATATCCATGCCATCC-3' (antisense). The PCR product was digested with BamHI/Xhol and ligated into BamHI/Xhol-cut pcDNA3 and pFlag-D to produce untagged and Flag-tagged expression vectors. All PCR-derived PKG constructs were sequenced.

**Cell culture and transfection**

293T cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum at 37 °C in a 5% \(\text{CO}_2\) atmosphere. The cells were transfected using Lipofectamine 2000 according to the manufacturer’s instructions (Life Technologies).

**RhoA and VASP phosphorylation in 293T cells and Western blotting**

293T cells were split into 12-well cluster dishes such that they would be 90–95% confluent 18 h later, at the time of transfection. The cells were transfected with expression vectors for wild-type or C43S-mutant PKG\(\alpha\) and RhoA or VASP as indicated in the figure legends. The next day, the wells were treated for 60 min with 250 \(\mu\)g 8-CPT-cGMP, 100 \(\mu\)M \(\text{H}_2\text{O}_2\), or vehicle as indicated. The medium was aspirated, and the cells were directly lysed by adding non-reducing SDS sample buffer (60 \(\text{mm}\) Tris-HCl, pH 6.8, 2% SDS, 0.01% bromphenol blue, and 100 \(\text{mm}\) maleimide (to prevent oxidation during sample processing)). Cell lysates were transferred to microcentrifuge tubes and sonicated 2 \(\times\) 20 s at 1-watt power. The proteins were separated by SDS-PAGE, transferred to Immobilon-P, blocked in 5% nonfat dry milk in TBS, and blotted with the indicated antibodies. The blots were developed using SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific).

**Protein expression and purification**

The cells were split into a 6-well cluster dish and transfected with Flag-tagged wild-type or C43S PKG\(\alpha\) (three wells each). Approximately 20 h post-transfection, the cells were scraped in PBS and lysed in buffer A (PBS, 0.1% Nonidet P-40) containing 1X protease inhibitor mixture (Calbiochem), and lysates were cleared by centrifugation (16,000 \(\times\) g, 10 min at 4 °C). Cleared lysates were incubated with 20 \(\mu\)l of anti-Flag M2 affinity gel (Sigma) for 1 h at 4 °C with constant mixing. The beads were washed 2 \(\times\) 200 \(\mu\)l of buffer A, 2 \(\times\) 200 \(\mu\)l of PBS with 500 \(\text{mm}\) NaCl, and 2 \(\times\) 200 \(\mu\)l of PBS. Bound proteins were eluted with 4 \(\times\) 10 \(\mu\)l of elution buffer (PBS with 100 \(\mu\)g/ml Flag peptide). For each elution step, the beads were incubated with buffer for 5 min on ice. The four eluates for each protein were pooled. Proteins were quantified by SDS-PAGE/Coomassie staining using BSA standards on the same gel. The gels were scanned, and quantification was performed using ImageJ.

**Kinase oxidation/reduction and in vitro kinase assays**

Flag-tagged wild-type and C43S PKG\(\alpha\) purified from transiently transfected 293T cells were diluted to \(\sim\)1 ng/\(\mu\)l in kinase dilution buffer (10 mM potassium phosphate, pH 7.0, 1 mM EDTA, and 0.1% BSA). For reduced samples, the dilution buffer contained 15 mM DTT. 10 \(\mu\)l of diluted kinase was added to 5 \(\mu\l\) of 3X kinase reaction mix (120 mM HEPES, pH 7.4, 1.5 mM Glastide, 30 mM MgCl\(_2\), 150 \(\mu\)M ATP, 180 \(\mu\)Ci/ml [\(\gamma\)-32P]ATP, and \(\pm\)30 \(\mu\)M cGMP). The reactions were performed for 1.5 min at 30 °C and stopped by spotting on P81 phosphocellulose paper. Unincorporated [\(\gamma\)-32P]ATP was removed by washing P81 paper four times for 5 min with 2 liters of 0.452% o-phosphoric acid. 32P incorporation was measured by liquid scintillation counting. In some experiments, 1.5 mM Glastide was replaced by 1.56 mg/ml Kemptide or 3 \(\mu\)g of histone H1. The reactions with histone H1 were run for 8 min. For experiments examining enzyme kinetics, reactions were performed \(\pm\)10 \(\mu\)M cGMP with increasing amounts of Glastide (0.005–1.0 \(\mu\)M) or with 1.5 mM Glastide in the presence of increasing concentrations of cGMP (0.003–10 \(\mu\)M).

**Data analysis**

The data were analyzed using GraphPad Prism 7. The \(V_{\text{max}}\) and \(K_m\) values were measured using non-linear fit Michaelis-Menten analysis, and cGMP \(K_m\) values were determined by plotting [agonist] versus normalized response with variable slope.

**Author contributions**—D. E. C. conceived the project. H. K., S. Z., and D. E. C. performed the experiments. R. B. P. and D. E. C. analyzed the data and wrote the paper.

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PKGIα is not activated by Cys43 disulfide bond formation