PKG\(\alpha\) is activated by metal-dependent oxidation \textit{in vitro} but not in intact cells

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Type I cGMP-dependent protein kinases (PKGIs) are important components of various signaling pathways and are canonically activated by nitric oxide– and natriuretic peptide– induced cGMP generation. However, some reports have shown that PKG\(\alpha\) can also be activated \textit{in vitro} by oxidizing agents. Using \textit{in vitro} kinase assays, here, we found that purified PKG\(\alpha\) stored in PBS with Flag peptide became oxidized and activated even in the absence of oxidizing agent; furthermore, once established, this activation could not be reversed by reduction with DTT. We demonstrate that activation was enhanced by addition of Cu\(^{2+}\) before storage, indicating it was reduced. In contrast, under the same conditions, purified PKGI\(\beta\) activity only slightly increased with storage. Using PKGI\(\alpha\)/PKGI\(\beta\) chimeras, we found that residues throughout the PKGI\(\alpha\)-specific autoinhibitory loop were responsible for this activation. To explore whether oxidants activate PKGI\(\alpha\) in H9c2 and C2C12 cells, we monitored vasodilator-stimulated phospoprotein phosphorylation downstream of PKGI\(\alpha\). While we observed PKGI\(\alpha\) Cys\(^{43}\) crosslinking in response to H\(_2\)O\(_2\) (indicating an oxidizing environment in the cells), we were unable to detect increased vasodilator-stimulated phosphoprotein phosphorylation under these conditions. Taken together, we conclude that while PKGI\(\alpha\) can be readily activated by oxidation \textit{in vitro}, there is currently no direct evidence of oxidation-induced PKGI\(\alpha\) activation \textit{in vivo}.

The type I cGMP-dependent protein kinases (PKGIs) play important roles in diverse physiological and pathophysiological processes. Their most studied and best understood signaling functions are in the cardiovascular system, where they control cardiac myocyte and smooth muscle contractility, but they also play key roles in synaptic plasticity, bone regulation, and beige/brown fat differentiation (1–3). As a result of differential splicing, mammalian cells express two PKGI isoforms, PKGI\(\alpha\) and PKGI\(\beta\), which have unique N-terminal leucine zipper and autoinhibitory domains, but identical cyclic-nucleotide binding and catalytic domains (4, 5). The unique N-terminal domains cause PKGI\(\alpha\) and PKGI\(\beta\) to form homodimers, target the kinases to different substrates, and cause PKGI\(\alpha\) to have a higher affinity for cGMP than PKGI\(\beta\) (6, 7). The higher cGMP affinity in PKGI\(\alpha\) correlates with a lower activation constant (\(K_a\)) for cGMP (6).

While the PKGI enzymes are canonically activated downstream of nitric oxide– and natriuretic peptide–induced cGMP generation, various groups have reported oxidation-induced direct activation of the kinase (3–7). The first report was by Landgraf \textit{et al.} (8), where the authors demonstrated that PKGI\(\alpha\) was activated by oxidation in the presence of various metal ions. Using tryptic digests and mass spectrometry, they identified Cys\(^{118}\), Cys\(^{196}\), Cys\(^{313}\), and Cys\(^{519}\) as the cysteines most likely mediating this effect. In 2007, Bugoyne \textit{et al.} (9) reported that PKGI\(\alpha\) could be activated by hydrogen peroxide (H\(_2\)O\(_2\))-induced disulfide formation between two cysteines at position 43 located at the C-terminal end of the leucine zipper/dimerization domain. However, we subsequently used cell-based and \textit{in vitro} kinase assays to demonstrate that disulfide formation at Cys\(^{43}\) does not lead to PKGI\(\alpha\) activation (10). We also found that the C43S mutation, which was generated to produce a “redox-dead” PKGI\(\alpha\), caused PKGI\(\alpha\) to have an approximately five-fold lower sensitivity to cGMP-induced activation \textit{in vitro}, compared to the WT enzyme (10). Our results were confirmed by Sheehe \textit{et al.} (11). In addition, Sheehe \textit{et al.} (11) concluded that H\(_2\)O\(_2\)-induced PKGI\(\alpha\) activation was due to conversion of Cys\(^{43}\) to sulfonic acid and proposed that the negatively charged sulfonic acid interacted with basic residues distal to the autoinhibitory sequence.

During our previous studies, we found that cGMP-independent basal activity of purified Flag-epitope–tagged PKGI\(\alpha\) increased after overnight storage in PBS with 100 ng/ml Flag peptide (Fig. 1). This activation occurred without the addition of an oxidizing agent and was associated with increased Cys\(^{43}\) crosslinking between the two peptide chains; however, while addition of DTT to the preactivated enzyme reversed Cys\(^{43}\) crosslinking, it did not reverse the increase in basal activity. The following studies were performed to probe the mechanism of PKGI\(\alpha\) activation, under these conditions, and to assess whether this activation mechanism is physiologically important.
Results

PKGα basal activity increases after overnight storage in Flag elution buffer

Freshly prepared Flag-tagged PKGα was diluted in KPE buffer alone or KPE with 5 or 15 mM DTT. Immediately before performing activity assays, samples of the diluted kinases were added to SDS sample buffer containing 100 mM maleimide, and the amount of Cys43-crosslinked PKGα was determined by nonreducing SDS-PAGE (Fig. 1A). The kinase was approximately 42% crosslinked in the absence of DTT and the crosslinking was almost completely reversed by DTT. We measured kinase activity on the diluted samples and found that, compared to the maximum cGMP-stimulated activity, basal activity was 4.3 ± 0.69% in the absence of DTT and 3.4 ± 1.2% or 2.7 ± 0.69% when incubated with 5 or 15 mM DTT, respectively (Fig. 1B). The slightly lower basal activity in the presence of DTT is similar to our previous results (10). The purified PKGα was then stored at 4 °C overnight in elution buffer (PBS + 100 μg/ml Flag peptide). The next day, aliquots were added to SDS sample buffer containing 100 mM maleimide, and the amount of Cys43-crosslinked PKGα was determined by Western blotting (Fig. 1C). The kinase activity was then measured on the diluted samples using an in vitro assay (Fig. 1D). The figure shows data from a single protein preparation with assays performed in triplicate. Similar results were observed with two independent protein preparations. PKGI, Type I cGMP-dependent protein kinase.

Figure 1. PKGα basal activity increases after overnight storage in Flag elution buffer. A, newly purified PKGα was incubated for 1 h on ice in KPE buffer with the indicated amount of DTT and the level of Cys43-crosslinked PKGα was determined by Western blotting (M = monomer, D = crosslinked dimer). B, kinase activity in the absence and presence of 10 μM cGMP was measured shortly after purification using an in vitro assay. C and D, the purified PKGα was stored overnight at 4 °C in elution buffer and then incubated for 1 h with the indicated amounts of DTT in KPE buffer. The amount of crosslinked PKGα with Cys43 oxidized was determined by Western Blotting (E) and kinase activity was measured (F). E and F, in vitro kinase activity of newly purified PKGα (E) and after overnight storage with different levels of dilution in PBS (F). The figure shows data from a single protein preparation with assays performed in triplicate. Similar results were observed with two independent protein preparations. PKGI, Type I cGMP-dependent protein kinase.
of the kinase were diluted in KPE buffer, with and without DTT, and kept on ice for 1 h. Western blots demonstrated that PKGia diluted in KPE in the absence of DTT was completely oxidized with ~75% migrating as a crosslinked dimer and ~25% running as an unknown oxidation product at a higher apparent molecular weight than the reduced monomeric protein (Fig. 1C). In the presence of 5 or 15 mM DTT, both oxidation products were reduced to ~40% or ~60% monomeric/reduced, respectively. The basal kinase activity was increased to a similar extent under all three conditions (Fig. 1D). These results are consistent with our previous finding that PKGia activity is independent of Cys43 crosslinking but demonstrates that the kinase is activated by some modification that is not easily reversed with DTT. Importantly, this modification occurred without adding H2O2 or other oxidizing agents to the purified protein (a second experiment with similar results is shown in Fig. S1).

It should be noted that the increase in basal kinase activity after overnight storage varied between different kinase preparations. This difference may be in part due to variable amounts of PKGia in each preparation and thus the ratio of protein to buffer during storage. To test this hypothesis, we purified PKGia and stored it overnight undiluted or diluted in elution buffer. As seen in Figure 1, E and F, the basal activity of a fresh PKGia preparation was 4.7 ± 0.80% and increased to 7.8 ± 0.32% after overnight storage when not diluted. However, when aliquots of this preparation were diluted to 2- and 5-fold before storage, the basal activity increased to 16 ± 1.4% and 39 ± 0.57%, respectively. Importantly, adding 2-fold more Flag peptide to the elution buffer had no effect on the increased activity, indicating that activation was not being mediated by the peptide (data not shown). Therefore, given the variability in the level of PKGia activation between protein preparations, all experiments in the main body of this article are from kinase reactions performed in triplicate on single protein preparations. To demonstrate qualitative reproducibility of the results, duplicate experiments using separate protein preparations are shown in Supplemental Data.

PKGia activation is prevented in the presence of reducing agents and metal chelators

Since short-term incubation with DTT did not reverse the kinase activation that had occurred during overnight storage, and metals have been shown to activate PKGia, we assessed whether activation could be prevented by adding either DTT or the metal chelator EDTA before overnight storage. As seen in Figure 2A, the basal activity of newly purified PKGia was 6.2 ± 0.34% and increased to 53 ± 0.97% after overnight storage in elution buffer alone, but in samples stored in elution buffer with DTT or EDTA, the increase in basal activity was largely prevented (6.0 ± 0.53 and 9.1 ± 0.58%, respectively) (Fig. 2B). To directly test the effect of heavy metals, we measured the basal activity of newly purified PKGia and then stored it overnight with and without added Cu2+. Basal activity of newly prepared kinase was 11 ± 1.6% of maximum and increased to 36 ± 0.62% versus 61 ± 2.2% after overnight storage in the absence or presence of added Cu2+, respectively (Fig. 2C). Taken together, these results are consistent with oxidation-induced activation being driven by the presence of trace metals in the storage buffer.

PKGia activation is independent of Cys43 oxidation

Even though Cys43 crosslinking was not directly responsible for PKGia activation, it is still possible that Cys43 plays role in the observed activation. Therefore, we compared activation between WT and C43S PKGia. The basal activity of newly purified WT and C43S PKGia were 4.6 ± 1.8 and 6.1 ± 3.2%, respectively (Fig. 3A). The amount of crosslinked WT PKGia was ~49% and as expected, no crosslinking was seen in the C43S mutant (Fig. 3B). After overnight storage, basal activity of WT and C43S PKGia increased to a similar extent, 33 ± 0.91 and 31 ± 0.50% of maximum activity, respectively (Fig. 3C). Similar results are shown in Fig. S3. The WT enzyme was completely crosslinked at Cys43 (Fig. 3D); however, it should be noted that the crosslinked WT and the monomeric C43S PKGia bands appeared as doublets, suggesting that oxidation events beyond Cys43 crosslinking were occurring. Similar doublets have been reported by Donzelli et al. (12) and are thought to be the result of disulfide bond formation between Cys118 and C196.

Prysyazhna et al. (13) reported that Cys43 crosslinking alters PKGia’s activation by cGMP; however, in a previous study, we found that Cys43 crosslinking had no effect on the K", for
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**Figure 3. PKGα activation is independent of C43S oxidation.** A, the activities of freshly purified WT and C43S PKGα were analyzed by in vitro kinase assays. B, Western blotting showing amount of Cys43-crosslinked PKGα in the two preparations immediately after purification (M = monomer, D = crosslinked dimer). C, in vitro kinase assays performed using the protein preparations from (A) after overnight storage in elution buffer. D, Western blot showing the amount of crosslinked PKGα after overnight storage. The figure data shows from a single protein preparation with assays performed in triplicate. Similar results were observed with two independent protein preparations. PKGI, Type I cGMP-dependent protein kinase.

cGMP (10). Other noncanonical cyclic nucleotides have been reported to activate PKGα (14, 15), and it is possible that Cys43 crosslinking could alter the affinity for these nucleotides. To test this possibility, we performed kinase reactions with increasing concentrations of cAMP, cCMP, and cIMP using oxidized and reduced PKGα (Fig. S4). We found that Cys43 crosslinking had no effect on the $K_a$ for any of these nucleotides.

**Mutation of either Cys118 or Cys196 reduces oxidation-mediated PKGα activation**

In order to determine if oxidation of PKGα Cys118 or Cys196 was responsible for the overnight activation, we used mutagenesis to change the cysteines to nonoxidizable residues. These residues are located in the first cGMP-binding pocket (Fig. 4A), and a disulfide bond was seen between these residues in a crystal structure of the isolated CNB-A/B domains [Fig. 4B and (16)]. Since we wanted to produce mutations that prevent oxidation-induced activation, but otherwise have no effect on basal kinase activity or cGMP response, we identified amino acid differences at these positions in homologous proteins, reasoning that changing the cysteines to these residues would be less likely to disrupt folding of the cGMP-binding pocket. Thus, we aligned PKGI, PKGII, and PKA R1α amino acid sequences and found that R1α has an alanine at the position analogous to Cys118 and that PKGI1 has a valine and R1α has a serine at the position analogous to Cys196 (Fig. 4C). Thus, we compared activation of WT, C118A, and C196V PKGα. As seen in Figure 4D, the basal activities of WT, C118A, and C196V were 5.9 ± 1.4, 5.0 ± 0.9, and 6.9 ± 1.1% of maximum, respectively. The next day, basal activities increased to 53 ± 1.9% of maximum for WT but only to 17 ± 1.8 and 35 ± 0.1% for the C118A and C196V mutant kinases, respectively (Fig. 4E). Similar results for a separate enzyme purification are shown in Fig. S5. While mutation of Cys118 had the most pronounced effect on preventing activation, the C196V mutation also reduced the level of activation. Together, these data demonstrate that in addition to oxidation of Cys118, oxidation of Cys196 and/or other residues can also induce PKGα activation.

**PKGβ is not highly activated by overnight oxidation**

Since PKGα and PKGβ have identical sequences in their first cyclic nucleotide-binding pockets (which contain both Cys118/Cys133 and Cys196/Cys311), we examined whether PKGβ is also activated during overnight storage. PKGα and PKGβ purified and immediately assayed showed a basal activity of 4.9 ± 1.2% and 1.6 ± 0.54% of maximum, respectively (Fig. 5A). After overnight storage at 4 °C, as expected, the basal activity of PKGα increased to 21 ± 1.3%, whereas the basal activity of PKGβ only slightly increased to 4.0 ± 0.78%. Similar results are shown in Fig. S6, A and B. These findings are consistent with those reported by Sheehe et al. (11), who showed that unlike PKGα, purified PKGβ was resistant to H2O2-induced activation.

**Testing the activation mechanism proposed by Sheehe et al.**

To explain the different response of PKGα and PKGβ to H2O2-induced activation, Sheehe et al. (11) proposed a mechanism in which basic residues unique to the PKGα autoinhibitory loop interacted with a negatively charged sulfonic acid moiety formed at Cys118 in response to H2O2. We tested this mechanism by mutating the basic residues found in the PKGα autoinhibitory loop to the corresponding nonbasic residues in PKGβ. Specifically, we simultaneously mutated PKGα Arg12 to Phe (R82F) and Lys83 to Pro (K83P). We found that in freshly purified preparations, the basal activity of the mutant protein (referred to as RK/FP) was similar to WT PKGα, and that the mutations did not prevent activation after overnight storage (Fig. 5, C and D). Similar results are shown in Fig. S6, C and D. These results are not consistent with the activation mechanism proposed by Sheehe et al., but suggest different mechanisms, tested below.

**Residues throughout the PKGα autoinhibitory region mediate oxidant-induced activation of PKGα**

Since overnight storage differentially affected the basal activities of PKGα and PKGβ, we made chimeric enzymes in which we swapped the leucine zipper domains between the two kinases (chimera C1, Fig. 6A). The α/β kinase had a PKGα leucine zipper with a PKGβ autoinhibitory domain and the β/α kinase had the opposite (the remaining sequences are identical between the two isoforms). We then performed
kinase assays on freshly purified PKGα, PKGβ, PKGαβ, and PKGβα and found that they had similar basal activities (Figs. 6B and S7A). After overnight storage, the basal activities of PKGα and PKGβ/α increased to a similar degree, but the basal activities of PKGβ and PKGαβ remained low (Figs. 6C and S7B). Thus, activation required residues in the PKGα autoinhibitory domain. To localize the residues responsible for activation, we made another set of complementary chimeric enzymes by swapping the amino acids N-terminal to the ISAEP amino acid sequence, which is conserved in both isoforms and located after the pseudosubstrate sequence in the autoinhibitory domain (chimera C2, Fig. 1A). After overnight storage, basal activity increased in both chimeric enzymes, but the increase was less than that seen for WT PKGα (Fig. 6, D and E). The same pattern of activation was seen with separate enzyme preparations (Fig. S7, C and D), suggesting that activation is most likely mediated through an additive effect involving residues throughout the PKGα autoinhibitory loop.

Testing the effect of acidic residue mutations at PKGα Cys\textsuperscript{118} and PKGβ Cys\textsuperscript{196} on kinase activity

Since Sheehe et al. demonstrated that H\textsubscript{2}O\textsubscript{2} treatment caused conversion of Cys\textsuperscript{118} to a negatively charged acid moiety which then induces kinase activation, we examined the effect of mutating Cys\textsuperscript{118} to Asp. We also assessed the corresponding mutation in PKGβ (i.e., C133D). Freshly purified C118D PKGα and C133D PKGβ had higher basal activities than the WT enzymes (Fig. 7A). The basal activities of both mutants further increased after overnight storage (Fig. 7B), indicating that the enzymes were activated by modification of one or more additional site(s). Separate enzyme preparations with similar results are shown in Fig. S8.
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H₂O₂ does not activate PKGα in cultured cells

The H9c2 cell line was originally derived from embryonic rat heart (17). The cell line expresses endogenous PKGα and vasodilator-stimulated phosphoprotein (VASP), a well-characterized PKGI substrate. To assess how H9c2 cells respond to cGMP-induced PKGα and PKGIβ immediately after purification, B, kinase assays performed after overnight storage in elution buffer. C, kinase assays performed with WT and R82F/K83P (RK/FP) PKGα within 1 h of purification. D, kinase assays performed on the protein preparations shown in C after 20-h storage in elution buffer at 4 °C. The figure shows data from a single protein preparation with assays performed in triplicate. Similar results were observed with two independent enzyme purifications. PKGI, Type I cGMP-dependent protein kinase.

Metal-induced activation of purified PKGα in vitro

The first description of PKGI regulation by oxidation was reported by Landgraf et al., who found that PKGI purified from bovine lung could be activated by incubation with various metals, including Ag⁺, Hg⁺, Cu⁺, Cu²⁺, and Fe³⁺. They also demonstrated that activation by Cu²⁺ was blocked by coincubation with the reducing agent DDT or the metal chelator EDTA (8). These results are consistent with our current findings, which suggest that trace metals in the buffers (or carried over from cell extracts during purification) induced PKGα activation during storage. These authors found that Cu²⁺-induced activation could be reversed by removing the Cu²⁺ by gel filtration and reducing the enzyme with DTT, and they concluded that activation was due to the formation of intrachain disulfide bond(s) between either Cys¹¹⁸:Cys¹⁹⁶ or Cys¹¹⁸:Cys¹³⁵: Cys³¹⁹. Consistent with this conclusion, Donzelli et al. (12) proposed that PKGα could be activated by nitroxyl-induced disulfide bond formation between Cys¹¹⁸ and Cys¹⁹⁶, and Osborne et al. (16) observed a disulfide bond between these residues in a crystal structure of the PKGα cyclic nucleotide-binding domains. In contrast to activation being induced by formation of a Cys¹¹⁸:Cys¹⁹⁶ disulfide bond, Shehee et al. found that H₂O₂-induced oxidation converted Cys¹¹⁸ to sulfonic acid and proposed that PKGα activation was caused by interaction between the newly formed acidic moiety and basic residues unique to the PKGα autoinhibitory domain. While our current results are consistent with the conversion of Cys¹¹⁸ to sulfonic acid, we found that mutation of the basic residues that were predicted to interact with the sulfonic acid moiety did not prevent PKGα activation.

Oxidant-induced PKGα crosslinking at Cys⁴³ does not increase kinase activity but may alter cellular targeting

In 2007, Burgoyne et al. (9) reported that PKGIα could be activated by oxidant-induced disulfide formation between which result in a high level of oxidant-induced PKGIα Cys⁴³ crosslinking, PKGIα is not activated in H9c2 or C2C12 cells.

Discussion

PKGIs play key roles in the cardiovascular system and are the indirect targets of a number of pharmacological agents that work by raising intercellular cGMP levels (18). While a number of studies over the last 30 years have provided a wealth of insight into PKGI regulation and signaling, new findings continue to emerge. These findings include noncanonical modes of kinase regulation, detailed descriptions of mechanisms of cellular targeting and compartmentalization, and new downstream substrates which regulate novel signaling pathways or cellular processes. One of the most interesting areas of study has been the direct regulation of PKGIα activity by oxidation, which remains controversial (19, 20). In this article, we show that while PKGIα is activated by oxidation in vitro, oxidation does not directly activate the kinase in intact cells. A mechanistic schema for the different ways oxidation affects the activity of purified PKGIα versus PKGα activity/signaling in intact cells is shown in Figure 9.
two cysteines at position 43 located at the end of the leucine zipper in each PKGα peptide in the homodimer. A knock-in mouse containing PKGα with a C43S mutation has a phenotype consistent with loss of PKGα function, which implied oxidation-induced PKGα activation was an important physiological mechanism for regulating the kinase (21–24). However, we and others have reported that Cys43 crosslinking does not increase PKGα kinase activity in vitro (10, 11). Importantly, we found that the ‘redox-dead’ C43S mutation caused PKGα to be 5-fold less sensitive to cGMP-induced activation (10). A decrease in cGMP sensitivity for C43S PKGα was also seen by Shehee et al. (11). The reduced cGMP affinity could theoretically explain the loss-of-function phenotype of the C43S PKGα knock-in mouse.

The PKGα leucine zipper domain is involved in mediating homodimerization of the enzyme and also targets the kinase to specific substrates (25–27). The importance for proper PKGα targeting in vivo has been demonstrated by a knock-in mouse with mutations in the leucine zipper that prevent dimerization. These mice show adult onset hypertension and are more sensitive to cardiac pressure overload than wild-type littermates (i.e., increased hypertrophy, systolic/diastolic dysfunction, and mortality) (28, 29). While PKGα is dimeric in the absence of Cys43 crosslinking, crosslinking may stabilize the helical conformation of the leucine zipper, especially at its C-terminus, and may confine the conformation of an interface for protein–protein interactions (30). Consistent with this, Cys43 crosslinking increases the interaction between PKGα

Figure 6. Residues throughout the PKGα autoinhibitory region mediate oxidant-induced activation of PKGα. A, domain maps of PKGα and PKGβ showing the locations of the chimeric splice sites (C1 and C2). The amino acid sequences of the chimeric proteins are shown below, with the splice sites indicated by hyphens. The location of the pseudosubstrate sequence is indicated by a black box. B, kinase assays performed on WT and leucine zipper swapped C1 chimeric PKGα within 1 h of purification (α = PKGα, β = PKGβ, α/β = chimeric protein with PKGα leucine zipper and PKGβ autoinhibitory loop). C, kinase assays performed using the protein preparations shown in panel B after 20-h storage in elution buffer at 4°C. D, kinase assays performed on WT and C2 chimeric PKGα within 1 h of purification (α = PKGα, β = PKGβ, α/β = chimeric PKGα with PKGβ residues N-terminal to the splice site). E, kinase assays performed using the protein preparations shown in panel D after 20-h storage in elution buffer at 4°C. Al, autoinhibitory loop; CNB, cyclic nucleotide binding domains; LZ, leucine zipper; PKG, Type I cGMP-dependent protein kinase.
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![Figure 7. Testing the effect of acidic residue mutations at PKGIA Cys118 and PKGIB Cys116 on kinase activity. A, Kinase assays using newly purified PKGIA, PKGIB, C118D PKGIA, and C133D PKGIB. Assays were performed within 1 h of purification. B, kinase assays performed on the protein preparations shown in A after 20-h storage in elution buffer at 4 °C. The figure shows data from a single protein preparation with assays performed in triplicate. Similar results were observed with two independent enzyme purifications. PKGI, Type I cGMP-dependent protein kinase.

and two of its known interacting proteins, MYPT1 and RhoA, in vitro (9).

H2O2 does not activate PKGIA in cardiac myocyte-derived H9c2 cells or C2C12 myoblasts

The H9c2 cell line derived from embryonic rat hearts has been used as an alternative to primary cardiac myocytes (17). The cell line expresses PKGIA and VASP and thus serves as an ideal platform to study PKGIA signaling in a cellular context. VASP phosphorylation is a sensitive readout for PKGIA activation, and treating these cells with cell-permeable cGMP analogs leads to robust VASP phosphorylation. However, we were unable to detect VASP phosphorylation after treatment with relatively high amounts of H2O2 in either H9c2 or C2C12 cells. While H2O2 is an endogenous signaling molecule, the amounts found in vivo are thought to normally be in the low μM range but may reach higher levels under pathophysiological conditions (31). The finding that oxidant-induced PKGIA activation in vitro is due to irreversible modification of cysteines to sulfenic and/or sulfonic acids strongly argues against it serving as a dynamic signaling mechanism in vivo.

H2O2 may increase PKGIA substrate phosphorylation by activating soluble guanylate cyclase or inhibiting phosphatases

If PKGIA is not activated by oxidation in intact cells, how are we to account for experiments showing that tissues from C43S knock-in mice are resistant to H2O2 induced relaxation, but still relax in response to cGMP-analogs and nitrovasodilators? Previous studies have shown that treatment with H2O2 can activate soluble guanylate cyclase (sGC) (32–34). This activation seems to require a reaction between H2O2 and superoxide to form hydroxyl radicals (32) or metabolism of H2O2 by catalase to form Compound I (33). However, sGC can also be inhibited by oxidation (35). Thus, treatment with H2O2 may transiently activate sGC and produce a localized pool of cGMP. In this case, relaxation would rely on properly localized PKGIA with a high sensitivity to cGMP which can respond to this pool. Under such conditions, the loss of cGMP affinity and/or mislocalization of C43S PKGIA could explain the failure of tissues from the knock-in mouse to relax in response to H2O2. It should be noted that H9c2 and C2C12 cells do not express sGC, since PKGIA is not activated in response to nitric oxide donors (data not shown).

An apparent increase in PKGIA activity may also be due to inhibition of serine/threonine phosphatases by H2O2. Humphries et al. (36) found that enhanced cAMP-dependent protein kinase (PKA) substrate phosphorylation, seen when HeLa cells are treated with the sulfhydryl-specific oxidant diamide, is blunted in the presence of phosphatase inhibitors, indicating that the enhanced phosphorylation is due to phosphatase inhibition rather than kinase activation. While the exact phosphatases affected were not identified, PP1 and PP2A are known to dephosphorylate the PKA substrate CREB (37, 38), which is also a substrate for PKGIA. Interestingly, Kim et al. (40) found that H2O2 treatment inhibits PP1 and PP2A in primary human diploid fibroblasts. Whether oxidant-induced phosphatase inhibition enhances PKGIA signaling in cells is currently unknown.

Study limitations and future directions

A limitation of this study is that in assessing the ability of oxidants to activate PKGIA in cells, we only examined one substrate (VASP) in two cell lines (H9c2 and C2C12). To analyze phosphorylation of other direct PKGIA substrates, we have tested a number of phospho-specific antibodies, but we found that they are not sensitive enough to detect substrate phosphorylation at endogenous protein levels in these cells. We have examined a number of primary cells and established cell lines, but we were unable to identify cells in addition to H9c2 and C2C12 cells which contain sufficient amounts of PKGIA without expressing sGC. Another
limitation of this study is that cell culture conditions may not reflect conditions found in vivo. It is possible that under certain pathophysiological conditions, which result in very high oxidant levels, PKGIα may become activated by oxidation-induced modification of Cys118 to an acid; but to our knowledge, there is no evidence that this modification occurs in cultured cells or in vivo. We are currently examining if Cys43 crosslinking changes PKGIα targeting in cells and the mechanism through which H2O2 may activate sGC.

Conclusion

In conclusion, the physiological significance of oxidation-induced PKGIα activation is doubtful. This is based on three main findings: (i) the observed in vitro oxidation is driven by metals in the presence of atmospheric oxygen; (ii) the activating modification is not easily reversed, arguing against a dynamic regulatory mechanism; and (iii) even in the presence of higher than physiological H2O2 levels, oxidant-induced PKGIα activation is not observed in cultured cells.

Experimental procedures

Materials

Fetal bovine serum, horseradish peroxidase (HRP)-conjugated anti-Flag M2 antibody, anti-Flag M2 affinity gel, and Flag peptide were from Sigma. Phospho-VASP (Ser239) Antibody was from Cell Signaling Technology. HRP-conjugated goat anti-mouse (115-035-062) and goat anti-rabbit (111-035-046)
antibodies were from Jackson Immuno Research. Kemptide was from AnaSpec, Inc. Cyclic nucleotide analogs were from BioLog Life Science Institute, and general laboratory reagents were from Fisher Scientific, Sigma Life Science, or Bio-Rad Laboratories.

**Vector constructs**

Flag-tagged WT PKGα, WT PKGβ, and C43S PKGα have been described previously (10). Additional mutations and chimeric PKGα/PKGβ were produced using overlapping extension PCR (41, 42). PCR products were digested with BamHI and XhoI and ligated into BamHI/XhoI cut pFlag-D (10). All constructs derived by a PCR step were sequenced.

**Cell culture and transfection**

HEK293T/17 (ATCC ACS-4500), C2C12 (ATCC CRL-1772), and H9c2(2-1) (ATCC CRL-1446) cells were grown at 37 °C in a 5% CO₂ atmosphere in Dulbecco’s Modified Eagle Medium supplemented with 10% fetal bovine serum. Cells were transfected using Lipofectamine2000 (Life Technologies) Medium supplemented with 10% fetal bovine serum. Cells were transiently transfected into HEK293T cells and 24 h later, expression vectors were added. For some experiments, puriﬁed PKGI was diluted to 10 μM ATP, 180 μM MgCl₂, 30 mM DTT, 5 mM EDTA, or 0.1% bovine serum albumin. For some reactions, KPEB control was diluted to an equal volume of PBS containing 100 mM maleimide. Lysates were cleared by centrifugation and aliquots were added to 3× SDS sample buffer with or without β-mercaptoethanol. Reduced samples were boiled at 100 °C for 5 min before loading on 9% SDS-PAGE gels. Nonreduced samples were loaded onto the gels without boiling. Western blots were performed as described above, using the indicated antibodies.

**Kinase purification**

Flag-tagged WT and mutant PKGα and PKGβ were pu- rified as described (10). Briefly, Flag-tagged expression vectors were transiently transfected into HEK293T cells and 24 h later, cells were lysed in buffer A [PBS, 0.1% NP40, and protease inhibitor cocktail (Calbiochem #539131)]. Lysates were cleared by centrifugation and incubated with anti-Flag beads for 1 h at 4 °C. Beads were extensively washed, and PKG was eluted in PBS with 100 μg/ml Flag peptide. Purified kinases were either used immediately or assayed after overnight storage at 4 °C in elution buffer (~20 h). For some samples, kinases were diluted with an equal volume of PBS containing a two-fold concentration of added reagents (i.e., 30 mM DTT, 5 mM EDTA, or 200 μM Cu(II)²⁺) before overnight storage.

**In vitro kinase assays**

Puriﬁed kinase was diluted to ~1 ng/μl in KPEB Buffer [10 mM potassium phosphate (pH 7.0), 1 mM EDTA, and 0.1% bovine serum albumin]. For some reactions, KPEB con- tained the amount of DTT indicated in the text, and the diluted samples were kept on ice for 1 h before the kinase reactions were performed. Dose/response reactions for non- canonical cyclic nucleotides were performed as described (10), using increasing concentrations of the indicated cyclic nucleo- tides. Cyclic nucleotide ⁴ᴷᵤ values were calculated and compared using GraphPad Prism 8. Reactions were initiated by adding 10 μl diluted kinase to 5 μl 3x kinase reaction mix [120 mM Hepes (pH 7.4), 30 mM MgCl₂, 180 μM ATP, 180 μCi/ml [γ-³²P] ATP, and 1.56 mg/ml Kemptide] with or without 30 μM cGMP. Kinase reactions were run for 1.5 min at 30 °C and stopped by spotting on P81 phosphocellulose paper. The P81 paper was washed four times in 2 l of 0.452% o- phosphoric acid, once in 95% EtOH, and dried in an 80 °C oven. Phosphate incorporation was determined by liquid scintillation counting.

**Western blotting for purified PKGI proteins**

Puriﬁed PKGI samples were diluted ~1:100 in KPEB buffer and mixed with 2:1 with 3× SDS-loading buffer containing 300 mM maleimide. Samples were loaded onto 9% SDS-PAGE gels without heating. Separated proteins were transferred to Immobilon, blocked with 5% milk in TBS. Blots were probed with HRP-conjugated anti-Flag antibody at a 1:5000 dilution in 5% milk.

**Analysis of VASP phosphorylation in H9c2(2-1) and C2C12 cells**

H9c2(2-1) and C2C12 cells were split into 12-well cluster dishes and 24 h later, wells were treated with 8-CPT-cGMP or H₂O₂ as indicated in the ﬁgure legends. Cells were lysed in ice cold Buffer A containing 100 mM maleimide. Lysates were cleared by centrifugation and aliquots were added to 3× SDS sample buffer with or without β-mercaptoethanol. Reduced samples were boiled at 100 °C for 5 min before loading on 9% SDS-PAGE gels. Nonreduced samples were loaded onto the gels without boiling. Western blots were performed as described above, using the indicated antibodies.

**Data availability**

All supporting data is in the article.

**References**


Oxidation activates PKGα in vitro but not in cells