Regulation of Nox1 Activity via Protein Kinase A-mediated Phosphorylation of NoxA1 and 14-3-3 Binding*

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Nox activator 1 (NoxA1) is a homologue of p67(phox) that acts in conjunction with Nox organizer 1 (NoxO1) to regulate reactive oxygen species (ROS) production by the NADPH oxidase Nox1. The phosphorylation of cytosolic regulatory components by multiple kinases plays important roles in assembly and activity of the phagocyte NADPH oxidase (Nox2) system, but little is known about regulation by phosphorylation in the Nox1 system. Here we identify Ser172 and Ser461 of NoxA1 as phosphorylation sites for protein kinase A (PKA). A consequence of this phosphorylation was the enhancement of NoxA1 complex formation with 14-3-3 proteins. Using both a transfected human embryonic kidney 293 cell Nox1 model system and endogenous Nox1 in colon cell lines, we showed that the elevation of cAMP inhibits, whereas the inhibition of PKA enhances, Nox1-dependent ROS production through effects on NoxA1. Inhibition of Nox1 activity was intensified by the availability of 14-3-3ζ protein, and this regulatory interaction was dependent on PKA-phosphorylatable sites at Ser172 and Ser461 in NoxA1. We showed that phosphorylation and 14-3-3 binding induce the dissociation of NoxA1 from the Nox1 complex at the plasma membrane, suggesting a mechanism for the inhibitory effect on Nox1 activity. Our data establish that PKA-phosphorylated NoxA1 is a new binding partner of 14-3-3 protein(s) and that this forms the basis of a novel mechanism regulating the formation of ROS by Nox1 and, potentially, other NoxA1-regulated Nox family members.

Although originally identified as a critical component of host defense by phagocytic leukocytes, it is now known that many cells generate intracellular reactive oxygen species (ROS) that have been shown to function as signaling molecules to mediate various biological responses. These include innate immunity, angiogenesis, cell growth and death, and a variety of pathogenic conditions (1–3). A family of NADPH oxidase (Nox) enzymes have been identified that are responsible for much of the ROS production observed in diverse tissues. The Nox proteins are directly related to the phagocyte NADPH oxidase (originally termed gp91(phox) and now Nox2), which generates superoxide as its primary reaction product. The Nox family is composed of Nox1, Nox2, Nox3, Nox4, Nox5, Duox1, and Duox2 (1, 2, 4). Nox1 is highly expressed in colon epithelium and in the colon tumor cell lines Caco-2, DLD-1, and HT-29 (5, 6) as well as to a lesser extent in a variety of other cell types (1).

The gastrointestinal epithelium serves a primary protective role against food irritants and commensal or pathogenic microbes. Disruption of innate immune responses in the gut causes persistent, chronic inflammatory disease. Often this can result in the development of inflammation-associated gastrointestinal tumors (7). Both the development of inflammation and tumor formation have been linked to the formation of ROS in diseased tissues (1). Nox1 appears to be one of the most likely sources for generation of these oxidants. Whether dysregulated Nox1 activity in these conditions results from increased stimulatory signaling or from the loss of modulating inhibitory signals is currently not known.

Nox1 is most closely related structurally to Nox2, and as with the phagocyte NADPH oxidase, superoxide generation by Nox1 requires the membrane subunit p22(phox) (8, 9). Nox1 activation also depends on interactions with regulatory subunits, including NoxO1, which is a p47(phox) adapter protein homolog, and NoxA1, a p67(phox) activator protein” homolog (10, 11). In addition, as with Nox2, full Nox1 activity is dependent on the Rac1 GTPase (12–14). The expression of Nox1 can be up-regulated by various factors, such as interferon-γ (15), BMP4 (16), Helicobacter pylori lipopolysaccharide (17), and platelet-derived growth factor and angiotensin II (18). Although up-regulation of Nox1 protein increases ROS generation, there is little information about the acute regulation of Nox1 activation. As with the phagocyte oxidase, phorbol esters (e.g. PMA) are able to stimulate Nox1 activity, although they are unlikely to do so through effects on NoxO1, which lacks the regulatory protein kinase C phosphatidylinositol 4,5-bisphosphate 3-kinase. This work was supported by Centers for Disease Control Grant CI000095 (to G. M. B. and U. G. K.), National Institutes of Health Grant CA068376 (to B. M. B.), and American Heart Association Award 0535081N (to B. A. D). 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. We dedicate this work to Bernie Babior, who helped in its initiation. † Senior co-author. To whom correspondence may be addressed: Emory University, Whitehead Bldg., Rm. 165, 615 Michael St., Atlanta, GA 30322. Tel.: 404-727-5880; Fax: 404-727-8538; E-mail: bdiebol@emory.edu.

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§ The abbreviations used are: ROS, reactive oxygen species; PKA, protein kinase A; Nox, NADPH oxidase; NoxA1, Nox activator 1; NoxO1, Nox organizer 1; PMAb, phorbol 12-myristate 13-acetate; HEK, human embryonic kidney; DPI, diphenyliodonium; IBMX, isobutylmethylxanthine; HA, hemagglutinin; GST, glutathione S-transferase; AngII, angiotensin II; Pipes, 1,4-piperazinediethanesulfonic acid; Fl, forskolin plus IBMX; WT, wild type; PKA-CA, PKA catalytic subunit; Rac1-CA, constitutively active Rac1(Q61L); CTx, cholelar toxin.

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phosphorylation sites controlling conformation-induced membrane targeting of p47\textsuperscript{phox} by PMA (10, 11, 19).

In the phagocyte Nox2 system, agonist-induced phosphorylation of p47\textsuperscript{phox} is a critical regulatory step in the translocation of the cytosolic oxidase components to the plasma membrane that leads to the assembly of the functional NADPH oxidase enzyme (20–25). In contrast, NoxO1 does not require phosphorylation for membrane binding but is constitutively membrane-associated due to the presence of the phosphoinositide-binding \textit{phox} domain (10, 11, 19). Consequently NoxA1 is also associated with the membrane under basal conditions due to its recruitment by NoxO1 (13). Membrane binding of NoxA1 may also be influenced by Rac1 under certain circumstances (13). Thus activation of Nox1 must involve some mechanism other than the phosphorylation-induced recruitment of the cytosolic regulatory components to form an active NADPH oxidase. Although the phosphorylation of p67\textsuperscript{phox} has been reported (26), the significance of such phosphorylation for NADPH oxidase function remains unknown.

Many studies have implicated cAMP elevation and the activation of its downstream mediator, protein kinase A (PKA), in the negative regulation of ROS production by phagocytes (27–29) and other tissues, although the mechanisms are not fully understood. For example, anthrax edema toxin inhibits ROS generation by human neutrophils via the cAMP/PKA pathway (27), and it has been reported that activation of PKA downregulates chemotactic peptide-induced oxidant production through the phosphorylation of p47\textsuperscript{phox} (30). However, chemotactic peptide activators of the neutrophil NADPH oxidase also stimulate cAMP formation (29), and this transient elevation of cAMP has been suggested to be required for normal oxidase activation (31). The inhibitory effects of cAMP and PKA on ROS production appear to be operative in other systems as well. Dopamine inhibits oxidative stress in vascular smooth muscle cells through activation of PKA (32), whereas adiponectin suppresses excess ROS production under high glucose conditions via a cAMP/PKA-dependent pathway in vascular endothelial cells (33).

14-3-3 proteins are a highly conserved, ubiquitously expressed protein family. In mammals, there are at least seven isoforms (\(\beta, \gamma, \epsilon, \zeta, \tau, \xi\), and \(\eta\)), each encoded by a distinct gene. 14-3-3 proteins form homo- and heterodimers that can interact with a wide variety of cellular proteins through specific phosphoserine/phosphothreonine-binding motifs (RSxpXP or RXXpSXp where pS is phosphoserine) (34–37). The effects of 14-3-3 proteins on their targets can be broadly defined as (i) inducing a conformational change in the target protein, (ii) masking a specific region on the target (this could include an active site, a ligand-binding region, or a region that interacts with another protein), and (iii) acting as a scaffold to regulate localization and/or substrate activity (38). Structural analysis has shown that each 14-3-3 protomer folds into an \(\alpha\)-helical structure with a conserved binding groove that accommodates phospho-Ser/Thr-containing sites (36, 39, 40) typically generated by basophilic kinases such as PKA and protein kinase B (41, 42).

In the present study, we showed that NoxA1 is a substrate both \textit{in vitro} and \textit{in vivo} for PKA. We identified two sites on NoxA1 that are phosphorylated by PKA and showed that phosphorylation results in the interaction of NoxA1 with 14-3-3\(\zeta\), which, in turn, leads to inhibition of Nox1 activity. We further established that the binding of 14-3-3\(\zeta\) to NoxA1 disrupts the plasma membrane localization of NoxA1 with Nox1 concomitant with inhibition of NoxO1 and Rac1 binding. Manipulation of cAMP levels and PKA activity in both the Nox1-containing CcD841 colon epithelial cell line and in the HT-29 colon adenocarcinoma line modulated ROS production in a manner consistent with negative regulation of NoxA1 by PKA and 14-3-3\(\zeta\). These findings identify a novel mechanism for the regulation of Nox1-mediated ROS generation.

**MATERIALS AND METHODS**

**Cell Culture and Reagents**—Human HEK293 cells, human CcD841T colon epithelial cells, and HT-29 colonic adenocarcinoma cells were maintained in Dulbecco’s modified Eagle’s medium (Invitrogen) containing 10% heat-inactivated fetal bovine serum (Invitrogen), 1 mm sodium pyruvate, 100 \(\mu\)M nonessential amino acids, and antibiotics (100 units/ml penicillin and 100 \(\mu\)g/ml streptomycin) at 37 °C in 5% CO\(_2\). The CcD841T cell line was derived from normal human colon epithelia and exhibits a normal epithelial cell (non-transformed) phenotype under the conditions of culture utilized (see ATCC catalog). Purified PKA catalytic subunit, diphenyliodonium (DPI), H89, forskolin, isobutylmethylxanthine (IBMX), horseradish peroxidase, lulinol, and monoclonal anti-Myc were purchased from Sigma. Polyclonal anti-NoxA1 antibody was generated in house as part of the Centers for Disease Control Program PO1 C1000095. Angiotensin II was from Bachem (Torrance, CA), monoclonal anti-HA-oxidase was from Roche Diagnostics, anti-PKA-specific Ser/Thr antibody was from Cell Signaling Technology (Beverly, MA), and polyclonal anti-actin antibody and radiochemicals were from MP Biomedicals (Solon, OH). ECL reagents were from Pierce, and Protein G- and Protein A-Sepharose beads were from Amersham Biosciences. Cholera toxin and cholera toxin B subunit were from Calbiochem.

**Plasmid Construction**—pcDNA3.1-Nox1 was from David Lambeth (Emory University), and human NoxO1 and NoxA1 were from Tom Leto (National Institutes of Health). pcDNA3.1-HA-PKA-CA was described previously (43). GST- or Myc-Nox1, -NoxO1, and -NoxA1 were from Michael Yaffe (Center for Cancer Research, Massachusetts Institute of Technology, Cambridge, MA). pcDNA3-HA-AT1 receptor was from Kevin J. Catt (National Institute of Child Health and Human Development, Bethesda, MD). pRK5-myc-Rac1CA-Q61L was described previously (43). GST- or Myc-NoxA1, -NoxO1, and -NoxA1 or GST-14-3-3\(\zeta\) was constructed by inserting the PCR product into the vector, pGEX4T3 or pRK5(Myc), respectively. GST-NoxA1 deletion mutants were amplified by PCR using pGEX4T3-NoxA1 as a template, and the following oligonucleotides as primers: 1–150–5’, CCGGAAATTCATGGCGCTCTCTTGAGGGACCTGGTGCGCGCC-3’; 1–150–3’, 5’-CCGCTCGAGCGTTAGGACATGCGCTCTCCCTTAGCTGC-3’.
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Measurement of Reactive Oxygen Species—Reactive oxygen was measured using luminol chemiluminescence as described previously (45). CCD841 cells or the transfected cells were cultured for 16 h (HEK293 cells) or 48 h (HT-29 cells) and harvested by incubation with trypsin/EDTA for 1 min at 37 °C. After being washed with phosphate-buffered saline (without MgCl₂ and CaCl₂), the cells were removed from the well, washed twice with cold Hanks’ balanced salt solution containing calcium and magnesium, then pelleted at 1000 × g for 5 min and resuspended in Hanks’ balanced salt solution. We used 5 × 10⁵ HEK293 cells per assay and 2 × 10⁵ HT-29 or CCD841 cells per assay. Chemiluminescence was measured for 30 min with or without stimulation by 2 μg/ml PMA or 100 ng/ml angiotensin II (AngII) at 37 °C. Nox1 activity was stimulated more than 2-fold in the presence of expressed constitutively active Rac1(Q61L) (Rac1-CA); this effect was not observed with equivalent expression of Rac1 wild type.

Membrane and Cytosol Fractionation—Cells grown in 10-cm dishes were treated with 1 mM vanadate for 1 h, washed once with cold 1× phosphate-buffered saline, and extracted in ice-cold lysis buffer (100 mM Pipes, pH 7.3, 100 mM KCl, 3.5 mM MgCl₂, 3 mM NaCl, 1 mM ATP supplemented with 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, and 1 mM vanadate) for 5 min. Cells were collected by scraping, and cell lysates were sonicated four times for 30 s. The homogenates were centrifuged at 3,000 rpm for 10 min to pellet nuclei and intact cells, and supernatants were then centrifuged at 45,000 rpm at 4 °C for 60 min to sediment plasma membranes. The cytosol-containing supernatant was removed, and the crude membrane pellet was gently washed with lysis buffer. Membrane fractions were resuspended in lysis buffer, sonicated three times for 30 s, and then centrifuged at 45,000 rpm at 4 °C for 60 min. Membrane and cytosol fractions were then assayed for total protein, and equal amounts (30 μg) were analyzed by Western blotting. For protein immunoprecipitation experiments, membrane fractions were solubilized with sonication in standard radioimmune precipitation assay buffer with 1% Nonidet P-40 and 0.1% SDS containing a protease inhibitor mixture for 1 h at 4 °C and then centrifuged at 45,000 rpm at 4 °C for 45 min. Individual proteins were immunoprecipitated using appropriate antibodies and analyzed by Western blotting as indicated in the figure legends.

Statistical Analysis—Values are given in this study as mean ± S.D. of at least three experiments. Statistically significant differences between sample groups were determined using the one-way analysis of variance test with a Dunnett and/or Bonferroni posttest. A p value of <0.05 was considered significant.

RESULTS

NoxA1 Is Phosphorylated by PKA—Upon examination of the amino acid sequence of NoxA1, we identified two potential phosphorylation sites (Ser₁⁷² and Ser₄⁶¹) based on the PKA consensus sequence represented by RX(S/T). To determine whether either Ser₁⁷² or Ser₄⁶¹ is phosphorylated by PKA,
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A, purified GST-tagged NoxA1, its mutants, or 2 μg of myelin basic protein (MBP) were subjected to in vitro phosphorylation in the presence (+PKA) or absence (−PKA) of PKA catalytic subunit with [γ-32P]ATP as described under “Materials and Methods.” The phosphorylated products were analyzed by SDS-PAGE and autoradiography (A, upper panels). Protein loading is shown by the Coomassie Blue-stained gels (lower panels). No phosphorylation was observed in the absence of PKA (rightmost side of A). B, phosphorylation of NoxA1 or its mutants (where SDA = S172A/S461A double mutant) by PKA was examined over 10–60 min in a reaction mixture containing 100 μM ATP, and the level of 32P incorporation was determined by β scintillation counting as described under “Materials and Methods.” C, HEK293 cells were transfected with Myc-NoxA1-WT, -NoxA1(S172A), -NoxA1(S461A), or -NoxA1(S172A/S461A) for 16 h and then treated with or without 50 μM forskolin plus 0.5 mM IBMX (FI) or 0.1 μM H89 for 60 min. Immunoprecipitation was carried out using antibody directed against Myc (NoxA1), and the presence of the phosphorylated NoxA1 and total immunoprecipitated NoxA1 was detected using anti-phospho-PKA-specific Ser/Thr antibody and anti-Myc antibody, respectively. Representative experiments from three separate experiments are shown, except for B in which the experiment was done twice.

mutants of NoxA1 were generated in which Ser172, Ser461, or both were changed to the non-phosphorylatable amino acid Ala by site-directed mutagenesis. In vitro kinase assays were performed using the purified catalytic subunit of PKA and either wild type GST-NoxA1 or its mutants as substrates, with myelin basic protein serving as a control. As shown in Fig. 1A, PKA phosphorylated the full-length NoxA1 wild type protein, as well as a degradation product running at a lower molecular weight. NoxA1 was not phosphorylated in the absence of PKA catalytic subunit (−PKA), and phosphorylation by PKA was reduced in the presence of the inhibitor H89 (not shown), indicating that the phosphorylation observed was PKA-dependent. Expressed fragments of NoxA1 indicated that phosphorylation occurred in two regions of the protein, within amino acids 151–250 containing Ser172 and within amino acids 301–476 containing Ser461, but was not detected in other portions of NoxA1 (data not shown). Consistent with these being the primary phosphorylation sites, mutation of Ser172 to Ala in peptide 151–250 and Ser461 to Ala in peptide 301–476 caused the loss of PKA-dependent phosphorylation of these fragments.

Analysis of the stoichiometry of NoxA1 phosphorylation by PKA in vitro showed that ∼0.5 pmol of [32P]ATP was incorporated per 1 pmol of intact wild type NoxA1 (Fig. 1B). Mutation of either the Ser172 site or the Ser461 site caused the loss of half (∼0.25 pmol) of the [32P]ATP incorporation, whereas mutation of both sites caused nearly complete loss of phosphate incorporation. Thus, about 25% of the recombinant NoxA1 is phosphorylated by PKA in vitro, indicating that NoxA1 is a reasonably good PKA substrate. These data also indicate that Ser172 and Ser461 represent the major sites for NoxA1 phosphorylation by PKA.

To verify that Ser172 and Ser461 of NoxA1 are phosphorylated by PKA in vivo, full-length NoxA1 wild type or its mutants were cotransfected into HEK293 cells, and intracellular cAMP levels were elevated by treating the cells with the adenylate cyclase-stimulating drug forskolin in the presence of the phosphodiesterase inhibitor IBMX. NoxA1 proteins were immunoprecipitated, and their phosphorylation state was determined by immunoblotting with an anti-phospho-Ser/Thr PKA-specific antibody. As shown in Fig. 1C, a very low level of basal in vivo phosphorylation of NoxA1 was detected that was largely
insensitive to the addition of the PKA inhibitor H89. Upon stimulation with forskolin plus IBMX (FI), NoxA1 phosphorylation was substantially increased. Mutation of S172A or S461A individually reduced the level of NoxA1 phosphorylation slightly. In contrast, the NoxA1(S172A/S461A) double mutant was not phosphorylated above the low background level in the presence of FI. The level of NoxA1 phosphorylation induced by FI was similar to that obtained upon expression of active PKA catalytic subunit (Fig. 1D), and the effect of FI was substantially reduced upon co-expression of a catalytically inactive PKA catalytic subunit (PKA-KD). Expression of PKA-KD alone did not increase the phosphorylation state of NoxA1. These results indicate that NoxA1 protein is phosphorylated on Ser\(^{172}\) and Ser\(^{461}\) in intact cells in response to the elevation of cAMP and activation of PKA.

Phosphorylated NoxA1 Is a New Binding Partner of 14-3-3 Protein—Analysis of the sequence surrounding the NoxA1 Ser\(^{172}\) phosphorylation site by Scansite software indicated it to be a potential 14-3-3 binding site (RGRS\(^{172}\)LP) (46). We therefore tested the hypothesis that NoxA1 phosphorylation by PKA might regulate the binding of 14-3-3 proteins to NoxA1. The glutathione-agarose-bound GST-NoxA1 protein was incubated with bovine brain lysate, and the bead-bound fraction was analyzed by Western blotting with anti-14-3-3 antibody, which detects a number of 14-3-3 family members present in bovine brain. We observed a specific interaction of NoxA1 with 14-3-3 family members in the presence of ATP and PKA (Fig. 2A). This interaction was reduced in the absence of ATP or after incubation of the ATP plus PKA-treated lysate with calf intestinal phosphatase.

We next evaluated the interaction of NoxA1 with HA-14-3-3\(^{3\xi}\) protein overexpressed in HEK293 cells. 14-3-3\(^{3\xi}\) minimally bound to GST-NoxA1 wild type, but this interaction was dramatically enhanced when GST-NoxA1 was prephosphorylated by PKA (Fig. 2B, left panel). Similarly the phosphomimetic GST-NoxA1(S172E/S461E) mutant strongly bound to 14-3-3\(^{3\xi}\) even in the absence of PKA, in contrast to the wild type protein or the non-phosphorylatable GST-NoxA1(S172A/S461A) protein (Fig. 2B, right panel). Analysis of the binding affinity of purified 14-3-3\(^{3\xi}\) for the phosphomimetic NoxA1(S172E/S461E) protein in vitro indicated a \(K_d\) of ~16 nm (supplemental Fig. 1).

Co-immunoprecipitation of NoxA1 and 14-3-3\(^{3\xi}\) was also observed in lysates from HEK293 cells expressing various Myc-NoxA1 wild type or mutant proteins (Myc-NoxA1(S172A/S461A) or -NoxA1(S172E/S461E)), HA-14-3-3\(^{3\xi}\), and HA-PKA (Fig. 2, C and D). More HA-14-3-3\(^{3\xi}\) was bound to NoxA1-WT in the presence of co-expressed active PKA catalytic subunit (PKA-CA) than in its absence. HA-14-3-3\(^{3\xi}\) strongly bound to Myc-NoxA1(S172E/S461E) either in the presence or absence of PKA-CA, whereas HA-14-3-3\(^{3\xi}\) failed to bind to Myc-NoxA1(S172A/S461A). NoxA1(S172A) or NoxA1(S461A) single mutants each exhibited slightly reduced 14-3-3\(^{3\xi}\) binding activity when PKA was present (data not shown). Similar results were obtained when either Myc-NoxA1 (Fig. 2C) or HA-14-3-3\(^{3\xi}\) (Fig. 2D) was the protein to which the immunoprecipitating antibodies were directed.

To verify that the binding of 14-3-3\(^{3\xi}\) to NoxA1 was dependent upon its ability to recognize phosphorylated sites on the NoxA1 protein, we prepared a K49E mutation in 14-3-3\(^{3\xi}\) that should no longer recognize the phosphorylated substrate (47). As shown in Fig. 2E, the 14-3-3\(^{3\xi}\)(K49E) mutant no longer effectively co-precipitated with NoxA1 in the presence of PKA-CA. These results establish that NoxA1 interacts with the 14-3-3\(^{3\xi}\) isoform and that this interaction is enhanced by the phosphorylation of NoxA1 at Ser\(^{172}\) and Ser\(^{461}\) by PKA.

Finally we examined the interaction of NoxA1 with endogenous 14-3-3\(^{3\xi}\) in the 293 cell system (Fig. 2F). Similar to the results obtained with exogenous expression, we observed endogenous 14-3-3\(^{3\xi}\) to specifically interact with NoxA1, and this interaction was dependent upon the phosphorylation of NoxA1 at Ser\(^{172}\) and Ser\(^{461}\) by PKA.

PKA-mediated Phosphorylation of NoxA1 and 14-3-3 Complex Formation Inhibit Nox1 Activity—To assess the significance of the PKA-mediated phosphorylation of NoxA1 and induced interaction with 14-3-3 protein(s) for Nox1 activity, we measured Nox1-dependent ROS production in transfected 293 cells using a chemiluminescence assay. These cells lack endogenous Nox1 components, and ROS production is totally dependent on the expression of Nox1, NoxA1, and NoxO1 (data not shown). Nox1 activity was stimulated with either PMA (Fig. 3A), which increases Nox1 activity through protein kinase C, or with AngII, which acts through the angiotensin I type receptor (Fig. 3B). PMA stimulated basal ROS production by more than 2-fold (Fig. 3A). This activity was fully sensitive to the NADPH oxidase flavoenzyme inhibitor DPI, supporting that it originated via a DPI-sensitive oxidase. Elevation of intracellular cAMP levels by treating the cells with forskolin in the presence of IBMX significantly inhibited the basal Nox1 activity but had little effect on PMA-induced activity. When PKA activity was elevated further by transfection of PKA-CA, there was a slight decrease overall in PMA-stimulated ROS production and a somewhat greater decrease in overall AngII-stimulated activity (Fig. 3B). This inhibitory effect was substantially enhanced when 14-3-3\(^{3\xi}\) was expressed, particularly in the presence of PKA-CA. In contrast, under basal conditions and stimulated conditions, the inhibition of PKA by inclusion of the PKA inhibitor H89 in the incubation enhanced Nox1-mediated ROS production and antagonized the inhibitory effects of forskolin-induced cAMP elevation, PKA-CA, and, particularly, 14-3-3 protein. A very similar pattern of effects was obtained overall when the AngII was used as a stimulus (Fig. 3B), although we note that the receptor-mediated AngII stimulation was more sensitive in general to inhibition by PKA activation.

We next sought to determine whether phosphorylation of the Ser\(^{172}\)/Ser\(^{461}\) sites on NoxA1 was necessary for inhibition of Nox1 activity by 14-3-3\(^{3\xi}\) (Fig. 3C). Overexpressed 14-3-3\(^{3\xi}\) significantly decreased basal and, to a lesser extent, active Rac1-CA-stimulated Nox1 activity in the presence of NoxA1 wild type or NoxA1(S172E/S461E), but it had no affect on Nox1 activity in the presence of NoxA1(S172A/S461A). Similarly Nox1 activity stimulated by the addition of PMA was also sensitive to inhibition by 14-3-3\(^{3\xi}\) in the presence of NoxA1-WT or NoxA1(S172E/S461E) phosphomimetic mutant but not in the presence of the non-phosphorylatable NoxA1(S172A/S461A)
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A

|        | GST | GST-NoxA1 |
|--------|-----|-----------|
| PKA    | -   | + + + + + + |
| ATP    | -   | + + + + + + |
| CIP    | -   | + + + + + + |
| 14-3-3| -   | + + + + + + |
| GST-NoxA1 |
| GST   |

B

Kinase reaction

| - - | + | + |
|-----|---|---|
| GST | GST-NoxA1-WT | GST-NoxA1-WT |
| Cell Lysate | GST | Cell Lysate |

C

| HA-PKA-CA | HA-14-3-3z |
|-----------|------------|
| WT | S172, 461A | S172, 461E |
| IP: anti-Myc | IB: anti-Myc |
| IB: anti-HA |

D

14-3-3z + PKA-CA

| WT | S172, 461A | S172, 461E |
| IP: anti-HA | IB: anti-Myc |
| IB: anti-HA |
| Cell lysates |

E

| HA-PKA-CA | Myc-NoxA1 | HA-14-3-3z-WT | HA-14-3-3z-K49E |
|-----------|-----------|---------------|----------------|
| + + + + + |                |               |               |

F

| Myc-NoxA1 | 14-3-3z |
|-----------|---------|
| + + + + + |        |

Cell lysates

HA-PKA-CA
Without HA-PKA-CA into the HEK293 cells for 16 h before cells were treated with or without 50 μM forskolin/IBMX decreased basal Nox1 activity (Fig. 4B). Although a decrease in the initial rate of ROS formation was evident, most of the inhibition resulted from a decrease in the extent of maximum ROS formation. Expression of additional 14-3-3z decreased in the extent of maximum ROS formation. Expression of additional 14-3-3z alone in the HT-29 cells produced a modest decrease in the maximum basal or Rac1-CA-stimulated ROS formation (Fig. 4, B and D). This effect was reduced in the presence of the PKA inhibitor H89 (Fig. 4, compare A versus B and C versus D), suggesting that the effect of 14-3-3z was dependent on a basal level of PKA activity in these cells. Significantly, upon elevation of cAMP levels by treatment with forskolin/IBMX or upon expression of PKA-CA, 14-3-3z further dramatically reduced Nox1 activity under all conditions (Fig. 4, A–D).

We observed similar regulatory effects of cAMP-elevating agents in CCD841 normal colon epithelial cells (Fig. 5A). FI markedly reduced the basal level of ROS production of CCD cells grown in serum. This inhibition was almost totally reversed in the presence of the H89 inhibitor. We also treated the cells with cholera toxin, the cAMP-elevating toxin generated by the Gram-negative bacterium Vibrio cholerae, which induces inflammation and diarrhea by invading the colon mucosal epithelium (48). Active cholera toxin (CTx) induced a concentration-dependent inhibition of ROS production by CCD841 cells that was not mimicked by the inactive membrane-binding B subunit (supplemental Fig. 2). At a concentration of active toxin that inhibited ROS formation to the same extent as with FI, the inhibition induced by CTx was reversed by the presence of H89, indicating that it was due to the activation of PKA resulting from cAMP elevation by the toxin (Fig. 5A). Coupled with the HT-29 cell observations, these data support the hypothesis that PKA and 14-3-3 are negative regulators of endogenous Nox1 in colon epithelia, likely acting through effects on Nox1 regulatory cofactor activity.

Phosphorylation of Nox1 and 14-3-3 Complex Formation Alters Membrane Localization of Nox1—Recently several studies have shown that plasma membrane localization of Nox1 is dependent on Nox1 and/or active Rac1 GTPase and that this association of Nox1 with the membrane is critical for Nox1 activity (12–14). One of the several functions of 14-3-3 proteins can be to modify or restrict the subcellular location of phosphorylated ligands (38). Therefore, we examined whether this might occur with Nox1 and be a potential mechanism for the inhibitory effects of PKA and 14-3-3ζ on Nox1 activity. In Nox1-, NoxO1-, and Nox1-coexpressing 293 cells, Nox1-WT and Nox1-(S172A/S461A) were localized in the membrane fraction (Fig. 6A). In contrast, a substantial portion of the phosphomimetic Nox1(S172E/S461E) mutant was localized to the cytosolic fraction. Treatment of cells with forskolin/IBMX increased the Nox1-WT protein level in the cytosolic fraction but failed to relocalize Nox1(S172A/S461A) to the cytosol (Fig. 6A). Co-treatment of cells with forskolin/IBMX in the presence of the PKA inhibitor H89 partially reduced forskolin/IBMX-mediated cytosolic relocalization of Nox1-WT while having little effect on the cytosolic localization of Nox1(S172E/S461E) (Fig. 6A). In the presence of 14-3-3ζ, wild type Nox1 was released into the cytosolic fraction, but this did not occur with the non-phosphorylatable Nox1(S172A/S461A) mutant (Fig. 6B). The additional inclusion of H89 decreased, whereas forskolin/IBMX increased localization of Nox1-WT to the cytosolic fraction (Fig. 6B). Similarly Nox1-WT release to the cytosol was slightly increased by 14-3-3ζ or PKA-CA individually but strongly increased by the combination of both (Fig. 6C). However, localization of Nox1(S172A/S461A) was not changed by 14-3-3ζ and/or PKA (Fig. 6C).

To further investigate the molecular basis for the loss of membrane association resulting from PKA-induced 14-3-3ζ phosphorylation of Nox1, we examined the subcellular localization of NoxA1, a subunit and ATP and then treated with or without 10 units of calf intestinal phosphatase (CIP) for 1 h at 30 °C. Non-phosphorylated or phosphorylated GST-proteins were then washed and incubated with bovine brain lysates (BBL). Non-phosphorylated or phosphorylated GST-proteins were then washed and incubated with bovine brain lysates (BBL). The loaded amount of GST-proteins was detected by anti-GST immunoblot. C. HEK293 cells were transiently transfected with or without Myc-Nox1A or its mutants, HA-14-3-3ζ (14-3-3ζ), and HA-PKA-CA. Cells lysates were immunoprecipitated with anti-Myc antibody, and anti-HA antibody was used to detect bound (immunoprecipitate (IP)) or total (Cell lysates) 14-3-3ζ or PKA-CA. Actin served as a loading control. D. As shown in A, Nox1-WT was not changed by 14-3-3ζ and/or PKA (Fig. 6C).

To further investigate the molecular basis for the loss of membrane association resulting from PKA-induced 14-3-3ζ phosphorylation of Nox1, we examined the subcellular localization of NoxA1, a subunit and ATP and then treated with or without 10 units of calf intestinal phosphatase (CIP) for 1 h at 30 °C. Non-phosphorylated or phosphorylated GST-proteins were then washed and incubated with bovine brain lysates (BBL). Non-phosphorylated or phosphorylated GST-proteins were then washed and incubated with bovine brain lysates (BBL). The loaded amount of GST-proteins was detected by anti-GST immunoblot. C. HEK293 cells were transiently transfected with or without Myc-Nox1A or its mutants, HA-14-3-3ζ (14-3-3ζ), and HA-PKA-CA. Cells lysates were immunoprecipitated with anti-Myc antibody, and anti-HA antibody was used to detect bound (immunoprecipitate (IP)) or total (Cell lysates) 14-3-3ζ or PKA-CA. Actin served as a loading control. D. As shown in A, Nox1-WT was not changed by 14-3-3ζ and/or PKA (Fig. 6C).
**FIGURE 3.** Nox1 activity is inhibited by PKA and 14-3-3 in HEK293 cells. A and B, pRK5-Myc-Nox1, -NoxO1, and -NoxA1 were cotransfected with pcDNA3-HA-14-3-3z (14-3-3z) and/or pcDNA3-PKA-CA into HEK293 cells for 16 h. Cells were pretreated with 50 μM forskolin plus 0.5 mM IBMX (FI), 0.1 μM H89, or 10 μM DPI for 1 h, and then ROS formation was determined by chemiluminescence assay. 100% of Nox1 activity is defined for coexpression of Nox1 + NoxO1 + NoxA1 (+ angiotensin I receptor (AT1R) for B) after stimulation with 2 μg/ml PMA (A) or 100 nM angiotensin II (B) in suspension and determination of ROS for 30 min. C, pRK5-Myc-Nox1, -NoxO1, and -NoxA1 or -NoxA1 mutants were cotransfected with or without pcDNA3-HA-14-3-3z and Rac1-CA in HEK293 cells, as indicated, for 16 h, and ROS formation was determined by chemiluminescence assay. 100% of Nox1 activity is defined for Nox1 + NoxO1 + NoxA1-WT coexpression in the absence of HA-14-3-3z. Equal protein expression levels for each experimental condition were verified by immunoblot (supplemental Fig. 3). The results shown represent the mean of triplicates of three separate experiments ± S.D. *, p < 0.05; **, p < 0.01.
binding, we evaluated the effect on the interaction of NoxA1 with NoxO1 and Rac1 in HEK293 cells. Fig. 6D shows that in the presence of forskolin/IBMX the binding of both NoxO1 and Rac1-CA to WT NoxA1 was inhibited. Binding was further reduced in the presence of 14-3-3ζ. In contrast, interaction with GST-NoxA1(S172A/S461A) was unaffected by the presence of FL or 14-3-3ζ. GST-NoxA1(S172E/S461E) exhibited the inability to effectively bind NoxO1 or Rac1-CA under all conditions.

As these regulatory components mediate the assembly of Nox1 with the membrane-bound Nox1 complex, our data suggest that the loss of these interactions as a result of NoxA1 phosphorylation and 14-3-3ζ binding likely prevents membrane recruitment of NoxA1.

We used the CCD841 and HT-29 cell systems to verify that similar regulation by PKA and 14-3-3 of the localization of NoxA1 occurred with endogenous protein. As expected from the above observations, we observed that forskolin/IBMX + 14-3-3ζ or PKA-CA + 14-3-3ζ induced the appearance of endogenous NoxA1 in the cytosol of HT-29 cells (Figs. 5B and 7, A and B). This was associated with the phosphorylation of NoxA1 (Fig. 5C) and the binding of 14-3-3 protein to NoxA1 (Figs. 5D and 7C) in both colon cell lines. In the CCD841 cells, treatment with CTx also induced the dissociation of NoxA1 from the membrane (Fig. 5B). This effect was reduced in the presence of H89 (Fig. 5B) and was correlated with the phosphorylation (Fig. 5C) and association of 14-3-3ζ (Fig. 5D) with NoxA1. Overall these data indicate that in both normal colon and colon cancer-derived epithelial cells the phosphorylation of endogenous NoxA1 by PKA induces the binding of 14-3-3 protein(s) and a decrease in NoxO1- and Rac1-dependent membrane association of NoxA1, leading to decreased Nox1-mediated ROS formation.

**DISCUSSION**

Nox1 is thought to play important roles in host defense against bacterial infection in the gut and to participate in signal transduction leading to cell proliferation and hypertrophy (7, 49–51). Consequently Nox1 contributes to the development of inflammatory disease of the colon as well as to the development and progression of colon cancers. Although highly homologous to the phagocyte NADPH oxidase Nox2, the regulation of Nox1 activation is less well understood. Full Nox1 activity has been shown to require the regulatory cofactors NoxO1, NoxA1, and Rac1 GTPase (1). In the presence of serum, cells transfected with Nox1, NoxO1, and NoxA1 produce significant levels of ROS that can be modestly enhanced by the phorbol ester PMA or by expression of an activated form of Rac1 GTPase (12–14). Nox1 has also been shown to be activated acutely through stimulation of the angiotensin type 1 receptor (18). To a large extent, however, the physiological regulators of Nox1 activity remain undefined.

In this study, we showed that Nox1 activity can be regulated by a CAMP/PKA inhibitory pathway. We identified two sites, Ser172 and Ser461, in NoxA1 that are phosphorylated by PKA both in vitro and in vivo (Fig. 1). The Ser172 site lies within a canonical 14-3-3 protein-binding domain (RRG5172Lp versus RXpSXP), and we observed that 14-3-3ζ was able to bind to Nox1 both in vitro and in vivo in a manner that was enhanced by PKA-mediated phosphorylation (Fig. 2). Interestingly, maximal 14-3-3 binding seemed to require phosphorylation of both Ser172 and Ser461 and was reduced by mutation of a site (K49E) in 14-3-3ζ that is required to recognize substrate phosphorylation motifs (47). The need for both phosphorylation sites on NoxA1 may reflect the known propensity for 14-3-3 dimers to interact with more than one phosphorylation site within a substrate to achieve stable binding (38, 52).
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We detected interactions of NoxA1 with 14-3-3 proteins in bovine brain lysates as well as in HEK293 cells and colon CCKD841 and HT-29 cells. Each of these biological sources contains 14-3-3-z by immunoblot (data not shown). We have not determined whether the interaction of NoxA1 is specific for 14-3-3-z versus other 14-3-3 proteins. However, in experiments with expressed 14-3-3-z protein, we did not observe significant phosphorylation-dependent binding to NoxA1, suggesting that not all 14-3-3 proteins are able to mediate this regulatory interaction.

The interaction of NoxA1 with 14-3-3-z has functional consequences for Nox1-dependent ROS formation. In the presence of PKA and 14-3-3-z protein, we observed substantial inhibition of Nox1-mediated ROS formation both in a transfected HEK293 cell model system (Fig. 3) and with endogenous components in the colon epithelial CCKD841 cell line and HT-29 colon carcinoma cells (Figs. 4 and 5). These inhibitory effects required the presence of phosphorylatable Ser172 and Ser461 residues in NoxA1. 14-3-3 protein induced some inhibition

FIGURE 5. CTx inhibits Nox1 activity in CCKD841 cells through PKA-mediated phosphorylation, 14-3-3 binding, and subcellular relocation of endogenous NoxA1. Attached CCKD841 cells were pretreated with or without 0.1 μM H89 for 1 h before incubation with or without 50 μM forskolin plus 0.5 mM IBMX (FI) for 1 h or 5 μg/ml CTx for 2 h (see supplemental Fig. 2). Cells were trypsinized and washed with phosphate-buffered saline, and then ROS formation was measured by chemiluminescence assay (A). B, cytosol and membrane protein levels of endogenous NoxA1 were determined by immunoblot (IB) analysis as under "Materials and Methods." C and D, cell lysates (500 μg) were immunoprecipitated with anti-NoxA1 antibody and anti-PKA Ser/Thr antibody used to detect phosphorylated NoxA1 (C) or anti-14-3-3-z antibody used to detect bound (immunoprecipitate (IP)) or total (cell lysates) 14-3-3-z (14-3-3-z) (D). Representative data from three separate experiments are shown. RLU, relative light units.

when expressed in cells alone; however, this effect was probably due to the presence of some phosphorylated NoxA1 under basal (+serum) conditions as it was reduced by the addition of the PKA inhibitor H89 (Figs. 3 and 4). Consistent with this, we observed slight increases in Nox1 activity in cells treated with H89 alone (Figs. 3 and 4). Inhibition by PKA and 14-3-3-z together was evident when Nox1 was activated by either PMA, Rac1(Q61L), or angiotensin II but was most effective with the receptor-mediated activation. This may be due to the fact that the first two stimuli represent non-physiological activating conditions. In particular, PMA would be expected to largely activate through stimulation of protein kinase C activity, although the mechanism by which protein kinase C might activate Nox1 remains unknown.

How does the phosphorylation of NoxA1 and the subsequent induction of complex formation with 14-3-3 module NoxA1 (and consequently Nox1) activity? We observed that under these inhibitory conditions there was substantial dissociation of NoxA1 from the membrane fraction where it normally is located. NoxA1 interacts through its C-terminal Src homology 3 domain with proline-rich repeat sequences in Nox1, as well as with Rac1 GTase through its N-terminal tetratricopeptide repeats (13, 19). These interactions are critical for Nox1 activation through the recruitment of the Nox1 activation domain to the membrane-localized Nox1. Because the Ser172 and Ser461 sites are near the Rac1-binding tetratricopeptide domain and Nox1-binding Src homology 3 domain, it is possible that the induced binding of 14-3-3 protein(s) to NoxA1 would sterically hinder these interactions. Importantly, because NoxA1 is required for Nox1 activity, a consequence would be to prevent the association of NoxA1 with the membrane-associated Nox1 complex, resulting in the inhibition of Nox1 activity. Indeed we observed that in the presence of forskolin plus IBMX the binding of NoxA1 to both Nox1 and Rac1 was markedly reduced (Fig. 6D). This loss of interaction with Nox1 and Rac1 was also constitutively observed with the Nox1(S172E/S461E) mutant, which mimics PKA-mediated phosphorylation to promote 14-3-3-z binding. Conversely, the binding of these regulatory partners to the non-phosphorylatable Nox1(S172A/S461A) mutant was unchanged by the presence of FI and/or 14-3-3-z. Whether
other kinases (and phosphatases) are able to modulate the activity of Nox1 or other Nox family members through a similar mechanism by regulating phosphorylation of the same or nearby sites on NoxA1 would be an interesting area for further investigation.

In summary, we identified a novel mechanism for the inhibitory regulation of stimulated Nox1-dependent generation of ROS that is mediated via a cAMP → PKA-mediated phosphorylation of the regulatory component NoxA1. We showed that NoxA1 is phosphorylated by PKA selectively at Ser172 and...
Ser-461 and that this phosphorylation induces the tight binding of 14-3-3 to NoxA1. Complex formation inhibited Nox1 activity via altered NoxA1 membrane localization in HEK293 and HT-29 cells as well as in CCD841 normal colon epithelial cells. These findings suggest that hormones or other agents able to stimulate cAMP formation and consequent PKA activation in cells with Nox1-dependent ROS formation would be susceptible to this inhibitory pathway. The hormones that participate in such pathways in the normal course of gut physiologically remain to be defined. Interestingly a number of bacterial...
toxins that may be generated by invading gut-localized bacteria (e.g. *V. cholerae* and *Bordetella pertussis*) are effective regulators of cAMP formation (48, 53, 54). As we show here for the cAMP-elevating cholera toxin (Fig. 5), the resulting increases in NoxA1 phosphorylation by PKA provide an effective mechanism for down-regulating ROS production and hence the normal innate immune response of the gastrointestinal epithelium.

PKA has been recently shown to undergo a redox-mediated enhancement of forskolin-stimulated kinase activity under mild oxidizing conditions (55). This appears to be a result of redox regulation of an inhibitory Ser/Thr phosphatase activity. Conversely at high levels of ROS, forskolin-stimulated PKA activity was inhibited due to direct oxidation of reactive thiol groups in PKA. Such biphasic regulation of PKA activity by ROS formation provides an intriguing self-limiting feedback cycle capable of controlling both Nox1 activity and PKA activity. Thus, the formation of ROS through early or low level Nox1 stimulation would act to enhance PKA activity. This would in turn feed back to phosphorylate NoxA1 and to subsequently reduce PKA activity and ROS formation. If conditions are such that the formation of ROS is very high, perhaps due to abnormally high Nox1 activity in intestinal disease or inflammation (e.g. as reported in Crohn disease (7)), then PKA would be effectively shut off, and Nox1 activity would be unchecked. Whether such a mechanism contributes to various inflammatory diseases of the colon and/or the formation of colon cancers remains to be investigated.

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