Angiotensin II inhibits the Na\(^{+}\)-K\(^{+}\) pump via PKC-dependent activation of NADPH oxidase

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White CN, Figtree GA, Liu CC, Garcia A, Hamilton EJ, Chia KK, Rasmussen HH. Angiotensin II inhibits the Na\(^{+}\)-K\(^{+}\) pump via PKC-dependent activation of NADPH oxidase. Am J Physiol Cell Physiol 296: C693–C700, 2009. First published February 4, 2009; doi:10.1152/ajpcell.00648.2008.—The sarcolemmal Na\(^{+}\)-K\(^{+}\) pump, pivotal in cardiac myocyte function, is inhibited by angiotensin II (ANG II). Since ANG II activates NADPH oxidase, we tested the hypothesis that NADPH oxidase mediates the pump inhibition. Exposure to 100 nmol/l ANG II increased superoxide-sensitive fluorescence of isolated rabbit ventricular myocytes. The increase was abolished by pretreated superoxide dismutase (SOD), by the NADPH oxidase inhibitor apocynin, and by myristilated inhibitory peptide to \(\epsilon\)-protein kinase C (\(\epsilon\)PKC), previously implicated in ANG II-induced Na\(^{+}\)-K\(^{+}\) pump inhibition. A role for \(\epsilon\)PKC was also supported by an ANG II-induced increase in coimmunoprecipitation of \(\epsilon\)PKC with the receptor for the activated kinase and with the cytosolic p47\(^{\text{phox}}\) subunit of NADPH oxidase. ANG II decreased electrogenic Na\(^{+}\)-K\(^{+}\) pump current in voltage-clamped myocytes. The decrease was abolished by SOD, by the gp91ds inhibitory peptide that blocks assembly and activation of NADPH oxidase, and by \(\epsilon\)PKC inhibitory peptide. Since colocalization should facilitate NADPH oxidase-dependent regulation of the Na\(^{+}\)-K\(^{+}\) pump, we examined whether there is physical association between the pump subunits and NADPH oxidase. The \(\alpha_\text{c1}\)-subunit coimmunoprecipitated with caveolin 3 and with membrane-associated p22\(^{\text{phox}}\) and cytosolic p47\(^{\text{phox}}\) NADPH oxidase subunits at baseline. ANG II had no effect on \(\alpha_\text{c1}\)/caveolin 3 or \(\alpha_\text{c1}/p22\(^{\text{phox}}\) interaction, but it increased \(\alpha_\text{c1}/p47\(^{\text{phox}}\) coimmunoprecipitation. We conclude that ANG II inhibits the Na\(^{+}\)-K\(^{+}\) pump via PKC-dependent NADPH oxidase activation.

Reduced nicotinamide adenine dinucleotide phosphatase oxidase; oxidant signaling; glutathionylation; protein kinase C; Na\(^{+}\)-K\(^{+}\)-ATPase

THE Na\(^{+}\)-K\(^{+}\) PUMP GENERATES transmembrane concentration gradients for Na\(^{+}\) and K\(^{+}\) that are essential for cell function. Its tight regulation is crucial. Although the pump is known to be regulated by a variety of hormones, the mechanisms are incompletely understood. Long-term regulation occurs via effects on gene expression, whereas short-term regulation typically is mediated by hormone receptors coupled to activation of protein kinases. However, phosphorylation sites on the pump molecule are poorly accessible (31), kinase activation is not necessarily associated with its phosphorylation (9), and the phosphorylation that does occur may be to a very low stoichiometry (8). The physiological role of phosphorylation of the Na\(^{+}\)-K\(^{+}\) pump subunits themselves in its regulation is therefore uncertain.

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MATERIALS AND METHODS

Cells. Single ventricular myocytes were isolated from male White New Zealand rabbits as described previously (20). A total of 44 rabbits were used for the study. The institutional review committee for animal research had approved experimental protocols. The myocytes were used on the day of isolation and stored at room temperature in Krebs-Henseleit buffer solution.

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Fluorescence microscopy. The oxidative fluorescent dye dihydroethidium (DHE) was used to image intracellular O$_2^-$ as we and others have previously described (21, 34). Myocytes were incubated in Krebs solution containing 2 μmol/l DHE for 20 min at 37°C in the dark. In some experiments they were preincubated in 170 IU/ml polyethylene glycol-conjugated superoxide dismutase (PEG-SOD), 10 μmol/l apocynin, or 10 μmol/l of myristolated εPKC inhibitory peptide for 20 min before loading with DHE. Myocytes were then exposed to control solutions or solutions containing 100 nmol/l ANG II for 10 min before fixation in 2% paraformaldehyde on ice for 4 min. They were washed and mounted on poly-L-lysine-coated glass slides in Vectashield and examined under a laser-scanning confocal microscope (Nikon C1) equipped with an argon-krypton laser. The excitation wavelength was 488 nm and the emission wavelength was 585 nm. The fluorescence images were obtained using constant settings of scanning speed, pinhole diameter, and voltage gain. Raw data from a representative z-section was saved and converted to a tiff file. All tiff files were imported into Adobe Photoshop 7.0 (Adobe Systems, San Jose, CA). Myocytes representative of each experimental condition were selected randomly for quantification of average fluorescence intensity after exclusion of the nucleus. Experimental conditions were coded by numbers, and data were decoded after analysis. Only myocytes with clear striations and rodlike shape were included in the analysis. The average intensity for cells from each experiment was normalized against its control (100%).

Measurement of electrogenic Na$^+$/K$^+$ pump current. We used the whole cell patch-clamp technique to measure electrogenic Na$^+$/K$^+$ pump current ($I_p$, arising from the 3:2 Na$^+$/K$^+$ exchange ratio). The superfusate contained (in mmol/l) 140 NaCl, 5.6 KCl, 2.16 CaCl$_2$, 1 MgCl$_2$, 10 glucose, 0.44 NaH$_2$PO$_4$, and 10 N-2-hydroxyethylpiperazine-N-$'$-2-ethanesulfonic acid (HEPES). It was titrated to a pH of 7.40 at 35°C with NaOH. This solution was used while establishing the whole cell configuration. Two to three minutes after the whole cell configuration was established, we switched to a superfusate that was nominally Ca$^{2+}$-free and contained 0.2 mmol/l CdCl$_2$ and 2 mmol/l BaCl$_2$. It also included ANG II when indicated. Myocytes were exposed to these solutions for ~10–12 min before $I_p$ was measured. In one series of experiments we replaced Na$^{+}$-containing compounds in the superfusate with N-methyl-$\beta$-d-glucamine (NMG, Cl) (16).

Wide-tipped patch pipettes (4–5 μm) were filled with solutions containing (in mmol/l) 5 HEPES, 2 MgATP, 5 ethylene glycol-bis(β-aminoethyther)-N,N,N’,N’-tetraacetic acid (EGTA), 70 potassium glutamate, 10 sodium glutamate, 80 tetramethylammonium chloride (TMA,Cl), and 0.01 l-arginine and were titrated to a pH of 7.20 at 35°C with KOH. Patch pipettes filled with these solutions had resistances of 0.8–1.1 MΩ. The series resistance after formation of the whole cell configuration had to be $\leq 2.8$ MΩ to satisfy previously defined criteria (36).

$I_p$ was identified at a holding potential of −40 mV as the difference between stable plateaus of holding current before and after Na$^+$/K$^+$ pump blockade with 100 μmol/l ouabain 10–15 min after the whole cell configuration was established. The electronic recording system and criteria for identification of stable currents and the ouabain-induced change in them have been reported previously (36). We report $I_p$ normalized for membrane capacitance and hence cell size. The effect of ouabain on $I_p$ is not reversible within the time frame stable holding currents can be reliably measured (10, 36). Separate myocytes were therefore used for measurements of $I_p$ for each set of experimental conditions.

Protein coimmunoprecipitation. Cells were lysed in ice-cold buffer containing 150 mmol/l NaCl, 50 mmol/l Tris-HCl (pH 8.0), 2 mmol/l EDTA, and 1% Triton X-100, and protease inhibitors were added. The lysates were clarified by centrifugation at 16,000 g for 20 min and were incubated with monoclonal antibody to the specific protein of interest or control IgGs. Protein A/G Plus-Agarose beads (40 μl of a 50% slurry) were added to the supernatant for a further incubation overnight at 4°C and were subsequently washed four times with PBS. Immunoprecipitated proteins were eluted by boiling at 95°C for 5 min in 40 μl Laemmli sample buffer. Immune complexes were analyzed by SDS-PAGE and Western blot by probing with antibodies to εPKC, the receptor for the activated kinase (εPKC RACK) p22$^{phox}$ and p47$^{phox}$, α$_1$-subunit of Na$^+$/K$^+$-ATPase, and caveolin 3.

Chemicals and reagents. TMA.Cl and NMG.Cl were purum grade and were obtained from Fluka Chemicals. All other chemicals used in Tyrode solutions were analytical grade and were obtained from BDH, Oaubain, apocynin, paraquat, CuZn SOD, PEG-SOD, and ANG II were obtained from Sigma. The gp91ds peptide was obtained from Sigma-Genosys Pharmaceuticals, Australia, and εPKC inhibitory peptide was obtained from Calbiochem. Myristolated εPKC inhibitory peptide was obtained from Biomol International. DHE was obtained from Invitrogen. Vectorshield was obtained from Vector Laboratories. Protein A/G Plus-Agarose beads as well as monoclonal antibodies to the p22$^{phox}$, and p47$^{phox}$ subunits of NADPH oxidase were obtained from Santa Cruz Biotechnology. Monoclonal antibody to the α$_1$-subunit of Na$^+$/K$^+$-ATPase was purchased from Upstate Biotechnology (clone C464.6), and antibodies to εPKC, εPKC RACK, and caveolin 3 were from BD Biosciences. Protease inhibitors (Complete EGTa-free) were obtained from Roche Diagnostics.

**RESULTS**

ANG II activates NADPH oxidase via εPKC in cardiac myocytes. We first examined whether ANG II increases fluorescence of cardiac myocytes loaded with the O$_2^-$-sensitive dye DHE. Exposure of myocytes to 100 nmol/l ANG II for 10 min increased DHE fluorescence compared with control. Representative micrographs are shown in Fig. 1A. To examine the specificity of this response and implicate the source of O$_2^-$, we preincubated myocytes in solutions containing 170 IU/ml of PEG-SOD or 10 μmol/l of the NADPH oxidase inhibitor apocynin. The increase in fluorescence was blocked by both PEG-SOD and apocynin. Since ANG II stimulates translocation of εPKC to the membrane in cardiac myocytes (28) and inhibits the pump in an εPKC-dependent manner (5), we examined the role of εPKC in the ANG II-induced increase in DHE fluorescence. Incubation of cardiac myocytes with 10 μmol/l of the membrane-permeable, myristolated εPKC inhibitory peptide abolished the ANG II-induced increase in fluorescence. The results of all fluorescence studies are summarized in Fig. 1B.

We also examined the role of εPKC in ANG II-induced activation of NADPH oxidase by a series of protein coimmunoprecipitation experiments in cardiac myocytes. ANG II increased the coimmunoprecipitation of the cytosolic p47$^{phox}$ subunit with the membranous p22$^{phox}$ subunit, consistent with translocation of p47$^{phox}$ and thus activation of NADPH oxidase (Fig. 2A). As shown in Fig. 2, B and C, ANG II also increased the coimmunoprecipitation of εPKC with its receptor for the activated kinase (RACK) as well as with p47$^{phox}$, which is a well-established substrate for PKC (27).
ANG II inhibits \(Na^+-K^+\) pump via NADPH oxidase

ANG II-induced \(Na^+-K^+\) pump inhibition is NADPH oxidase dependent. To examine the effect of ANG II on \(Na^+-K^+\) pump activity, we exposed patch-clamped myocytes to a control superfusate or a superfusate containing 100 nmol/l ANG II. We switched to the ANG II-containing superfusate after the whole cell configuration had been established. Fig. 3A shows the timing of changes in the composition of superfusates. Fig. 3B shows holding currents of a control myocyte and a myocyte exposed to ANG II before and after exposure to ouabain. The ouabain-induced shift in holding current, defining \(I_p\), was smaller for the myocyte exposed to ANG II than for the control myocyte. The mean \(I_p\) values for control myocytes and myocytes exposed to ANG II are shown in Fig. 3C. ANG II induced a significant decrease in \(I_p\).

While wide-tipped patch pipettes provide good control of intracellular \(Na^+\) (36), the electrochemical driving gradient for \(Na^+\) is inward, and ANG II might, in principle, increase \(Na^+\) influx and hence the intracellular \(Na^+\) concentration. This influx would stimulate the \(Na^+-K^+\) pump and cause an underestimation of the ANG II-induced decrease in \(I_p\). To examine whether such an underestimation is likely and to determine whether ANG II induces a decrease in intrinsic \(Na^+-K^+\) pump current with an independent set of data, we measured \(I_p\) in \(Na^+\)-free superfusates. Results are included in Fig. 3C. ANG II induced a decrease in mean \(I_p\) similar to the decrease measured in the \(Na^+\)-containing superfusate. The normalized holding currents recorded after exposure of myocytes to ouabain using \(Na^+\)-free or \(Na^+\)-containing superfusates in all experiments summarized in Fig. 3 were similar, indicating that ANG II did not induce a detectable change in non-pump membrane current.

Since ANG II activates NADPH oxidase (14) and increases \(O_2^-\)-sensitive DHE fluorescence in cardiac myocytes (Fig. 1), we examined the effect of including 200 IU/ml SOD in patch pipette solutions on the ANG II-induced pump inhibition. Superfusates contained \(Na^+\). The mean \(I_p\) values for control myocytes and myocytes exposed to ANG II are shown in Fig. 4. Perfusion of the intracellular compartment with pipette solution containing SOD abolished the ANG II-induced decrease in \(I_p\).

We next examined the dependence of ANG II-induced pump inhibition on NADPH oxidase. In one series of experiments we included 10 μmol/l apocynin in patch pipette solutions. Mean \(I_p\) values are included in Fig. 4. Apocynin abolished the ANG II-induced decrease in \(I_p\). In a second series of experiments we included 10 μmol/l of the gp91ds peptide in patch pipette solutions to inhibit docking of \(p47^{phox}\) and hence activation of NADPH oxidase. The gp91ds peptide is usually considered a low-efficacy inhibitor when combined with the \(tet\) peptide to facilitate transmembrane entry into intact cells (3). However, the whole cell patch-clamp technique allowed us to directly perfuse the intracellular compartment with a \(tet\)-free gp91ds peptide. Mean \(I_p\) values for control myocytes and myocytes exposed to ANG II are summarized in Fig. 4. The gp91ds peptide abolished the ANG II induced decrease in \(I_p\).

To examine the role of εPKC in the ANG II-induced decrease in \(I_p\), we included 100 nmol/l of εPKC inhibitory peptide in patch pipette solutions. Mean \(I_p\) values for control myocytes and myocytes exposed to ANG II are summarized in Fig. 5. The εPKC inhibitory peptide abolished the ANG II-induced decrease in \(I_p\). Since oxidative signaling might in principle activate PKC, the ANG II-induced decrease in \(I_p\) might be due to \(O_2^-\)-dependent activation of PKC and downstream phosphorylation of the \(Na^+-K^+\) pump itself or its associated molecules. To examine this, we included εPKC inhibitory peptide in patch pipette solutions, and we induced oxidative stress with paraquat. Myocytes were not exposed to ANG II. If oxidant-dependent pump inhibition were due to activation of PKC, we would expect paraquat-induced pump inhibition to be abolished by the inhibitory peptide. The peptide had no effect on the paraquat-induced decrease in \(I_p\), as shown in Fig. 6.

NADPH oxidase coimmunoprecipitates with the \(Na^+-K^+\) pump. Since our patch-clamp experiments implicated \(O_2^-\) generated by NADPH oxidase as the mediator of ANG II-induced pump inhibition, we next performed a series of protein coimmunoprecipitation experiments to investigate interactions between the \(Na^+-K^+\) pump and NADPH oxidase subunits. Total cell lysate from myocytes was immunoprecipitated with antibody to the pump's α, subunit, the most abundant isoform.

Fig. 1. Effect of ANG II on myocyte \(O_2^-\)-sensitive dihydroethidium (DHE) fluorescence. A: confocal fluorescence micrographs of a control myocyte and a myocyte exposed to ANG II. B: mean DHE fluorescence intensity of myocytes exposed or not exposed to ANG II. ANG II increased the fluorescence intensity. This increase was abolished by polyethylene glycol-conjugated superoxide dismutase (PEG-SOD), apocynin, or the εPKC inhibitory peptide. *Significant difference compared with control.
in cardiac myocytes (23). Immunoblotting was then performed with antibodies to two subunits of NADPH oxidase (NOX2): p22^phox and p47^phox. The results are shown in Fig. 7. The p22^phox subunit coimmunoprecipitates with the α1-subunit of the Na^+-K^+ pump. The association was unaffected by exposure to ANG II. The p47^phox subunit also coimmunoprecipitates with the α1-subunit of the pump under basal conditions. Consistent with translocation of p47^phox to the microdomain of the Na^+-K^+ pump, the degree of p47^phox and α1-coimmunoprecipitation was increased by exposure to ANG II. This was evident after 5 min of exposure, but it increased further after 10 min of exposure, as shown in Fig. 7B. Caveolin 3 has previously been shown to coimmunoprecipitate with both α1- and β1-pump subunits in rat cardiac myocytes where most Na^+-K^+ ATPase activity was concentrated in the caveolar membranes (25). Figure 7A shows that α1-pump subunit and caveolin 3 also coimmunoprecipitate in rabbit cardiac myocytes.

**DISCUSSION**

We examined the role of oxidative signaling in PKC-dependent ANG II-induced Na^+-K^+ pump inhibition in cardiac myocytes. The study of oxidative signaling is challenging. Detection of reactive oxygen/nitrogen species has limited specificity as have the pharmacological compounds used to scavenge them or used to inhibit the enzymes that generate them (38). Similarly, pharmacological PKC inhibitors/activators are imperfect, and there are technical difficulties in the study of Na^+-K^+ pump function in cardiac myocytes. We used different experimental approaches that in combination demonstrate that NADPH oxidase is critical for ANG II-induced pump inhibition.

The SOD-sensitive increase in DHE fluorescence of myocytes exposed to ANG II and the effect of SOD to abolish the ANG II-induced pump inhibition implicate O_2^-• in the pathway linking ANG II receptors to the Na^+-K^+ pump. The effects of apocynin to abolish the increase in fluorescence and the decrease in I_F suggest that NADPH oxidase is the source of O_2^-•. However, apocynin and all other pharmacological blockers have limited specificity for NADPH oxidase (3). We therefore also used the gp91ds peptide, designed to inhibit NADPH oxidase by mimicking its docking sequence with the p47^phox subunit. Perfusion of the intracellular compartment with pipette solution containing 10 μmol/l of gp91ds peptide abolished the ANG II-induced decrease in I_F in good agreement with an IC50 in the low micromolar range for NADPH oxidase inhibition in cell-free systems (29). The NOX family of oxidases is also implicated in the pump inhibition by the coimmunoprecipitation data. The catalytic α1-Na^+-K^+ pump subunit coimmunoprecipitated with the membrane-associated p22^phox NADPH oxidase subunit. There was no effect of ANG II on the coimmunoprecipitation. In contrast, ANG II induced an increase in the coimmunoprecipitation of α1 with the cytoplasmic p47^phox subunit that is translocated to the membrane with activation of NADPH oxidase.

Taken together, the studies on O_2^-•-sensitive DHE fluorescence, Na^+-K^+ pump currents and the coimmunoprecipitation...
implicate the NOX family of oxidases in the ANG II-induced pump inhibition. Of these, NOX2 and NOX4 are expressed in cardiac myocytes (26). Since NOX4 does not seem to require the p47phox subunit for activation (22), the role of p47phox implicated by our study suggests that NOX2 mediates the ANG II-induced Na\textsuperscript{+}-K\textsuperscript{+} pump inhibition. However, experimental tools used in assigning specific members of the NOX family to cellular locations and tasks have limited reliability (26), and a firm conclusion about the specific NOX that mediates the pump inhibition cannot be made.

Fig. 3. Effect of ANG II on Na\textsuperscript{+}-K\textsuperscript{+} pump current. A: timing of changes in the composition of superfusates. Arrow at left indicates establishment of the whole cell configuration and hence perfusion of the intracellular compartment with pipette solution. The switch from a Ca\textsuperscript{2+}-containing, ANG II-free solution in the tissue bath to a nominally Ca\textsuperscript{2+}-free solution containing Ba\textsuperscript{2+}, Cd\textsuperscript{2+}, and ANG II and the switch to a solution also containing ouabain (arrow at right) are shown. B: examples of holding current before and after exposure to ouabain. The ouabain-induced shift in holding current, $I_0$, was defined with the aid of an electronic cursor. $C_m$ indicates membrane capacitance in pF. C: mean $I_0$ normalized for membrane capacitance measured in superfusates containing Na\textsuperscript{+} at concentrations ([Na\textsuperscript{+}]) indicated (in mmol/l). Number of myocytes in each group is indicated in parentheses. *Significant difference between means of $I_0$.

Fig. 4. Role of superoxide and NADPH oxidase in ANG II-induced Na\textsuperscript{+}-K\textsuperscript{+} pump inhibition. Myocytes were exposed to superfusates as shown in Fig. 2A, and they were perfused with pipette solutions containing no inhibitor (control), SOD, apocynin (Apo), or the gp91ds peptide as indicated. Inhibition of ePKC abolished ANG II-induced Na\textsuperscript{+}-K\textsuperscript{+} pump inhibition. Number of myocytes in each group is indicated in parentheses. *Significant difference between means of $I_0$.

Fig. 5. Role of ePKC in ANG II-induced Na\textsuperscript{+}-K\textsuperscript{+} pump inhibition. Myocytes were exposed to superfusates as shown in Fig. 2A, and they were perfused with pipette solutions containing no inhibitor (control) or ePKC inhibitory peptide as indicated. Inhibition of ePKC abolished ANG II-induced Na\textsuperscript{+}-K\textsuperscript{+} pump inhibition. Number of myocytes in each group is indicated in parentheses. *Significant difference between means of $I_0$.

Fig. 6. Effect of PKC inhibition on oxidative regulation of the Na\textsuperscript{+}-K\textsuperscript{+} pump. Inclusion of 100 \mu mol/l of paraquat decreased $I_0$. Perfusion of myocytes with solutions that included ePKC inhibitory peptide had no effect on the paraquat-induced Na\textsuperscript{+}-K\textsuperscript{+} pump inhibition compared with that with no inhibitor (control). Number of myocytes in each group is indicated in parentheses. *Significant difference between means of $I_0$. 
The primary product generated by NADPH oxidases is $\text{O}_2^-$. It has limited access to candidate protein thiols, and its interaction with them is too slow to compete with its interaction with nitric oxide (NO) to form $\text{ONOO}^-$ or with its catalyzed dismutation to $\text{H}_2\text{O}_2$ (39). $\text{H}_2\text{O}_2$ has been implicated in biological effects of ANG II-induced activation of NADPH oxidase. However, modeling of kinetics indicates that, with the exception of peroxiredoxins, $\text{H}_2\text{O}_2$ and target thiol groups in proteins interact minimally at their typical cellular concentrations (38). This, in combination with complex ambiguities in interpretation of experimental data, makes it uncertain if $\text{H}_2\text{O}_2$ is a second messenger (39). Kinetic details of the interaction of $\text{ONOO}^-$ with cellular thiols are sparse, but the interaction is apparently fast enough to compete with its decomposition (39), and $\text{ONOO}^-$ has been implicated in the oxidation of target proteins (13).

Regulation of protein function by oxidative signaling depends on downstream molecular effects that are stable, yet reversible. Of the known oxidative modifications of proteins, glutathionylation fulfills these criteria (13). Further work is needed to examine for glutathionylation and other forms of oxidative modification of pump subunits and associated FXYD proteins. Oxidative signaling is critically dependent on proximity of sources and targets of the oxidant species (39). Such proximity in a caveolar domain is believed to be important for ANG II-induced, NADPH oxidase-dependent signaling in vascular smooth muscle (33). A high concentration of $\text{Na}^+\text{-K}^+$ pumps in cardiac myocytes caveolae (25), the coimmunoprecipitation of pump and NADPH oxidase subunits in this study, and localization of activated εPKC in cardiac myocyte caveolae (30) suggest that caveolar domain is also important for ANG II-induced $\text{Na}^+\text{-K}^+$ pump inhibition. Since NO synthase (NOS) is also localized in caveolae (2) and can mediate receptor-coupled $\text{Na}^+\text{-K}^+$ pump stimulation (35), opposing effects of NOS and NADPH oxidase may fine tune pump activity. However, this simple scheme may be complicated by an interaction with other sources of oxidant species because $\text{O}_2^-$ generated by NADPH oxidase can act as “kindling” for further $\text{O}_2^-$ synthesis by the uncoupling of NOS (3) and by the activation of xanthine oxidase (24).

Since it is firmly established that ANG II induces PKC-dependent phosphorylation of p47$\text{phox}$ and subsequent activation of NADPH oxidase (37), the widely accepted role of PKC in $\text{Na}^+\text{-K}^+$ pump regulation is easily retained by the scheme we propose. However, the effect of PKC to mediate pump inhibition contrasts with PKC-dependent increase in pump activity observed in mouse ventricular myocytes (15). In our studies, a specific isoform (εPKC) was implicated by its role in the activation of NADPH oxidase demonstrated in coimmunoprecipitation experiments and by the use of an isoform-specific inhibitory peptide in functional studies. In the mouse studies, PKC was implicated by activation of PKC with the phorbol ester phorbolester 12,13-dibutyrate (PDBu) (15). Phorbolesters can bind to receptors other than the PKC family of enzymes. However, nonspecific effects of PDBus are unlikely to account for the discrepancy because we have previously found that the effects of PKC activation with a phorbol ester are similar to both the effects of a nonspecific PKC-activating peptide and the effects of εPKC-activating peptide (δεRACK). All induced $\text{Na}^+\text{-K}^+$ pump inhibition (5, 6, 19). It is also difficult to reconcile our results with PKC-dependent pump regulation attributed to phosphorylation of the pump-related FXYD pro-
tein, phospholemman (4, 9, 12, 15) because the ANG II-induced, PKC-dependent pump inhibition was abolished by SOD and the gp91ds peptide, compounds not expected to inhibit PKC.

Although this study was performed on patch-clamped, internally perfused myocytes, our previous studies indicate that ANG II also inhibits the pump in isolated intact myocytes and in vivo (17, 19, 20). We treated rabbits with an angiotensin-converting enzyme inhibitor (ACEI) or an ANG II type I receptor (AT1) antagonist for 1 wk, isolated myocytes, and measured $I_p$. Inhibition of ANG II signaling in vivo was associated with an increase in pump activity in isolated patched-clamped myocytes and a decrease in the intracellular Na$^+$ concentration measured in isolated papillary muscles (20). Exposure of myocytes, from rabbits treated with ACEI, to ANG II before (6, 19) or after (5) the whole cell configuration was established decreased $I_p$. The increase in $I_p$ induced by treatment with ACEI or AT1 antagonist and the decrease in $I_p$ induced by in vitro exposure to ANG II were only detectable when K$^+$ was included in pipette solutions. (6). These studies suggest that the findings of the present study have relevance in vivo.

In conclusion, this study identifies a key role for oxidative signaling in regulation of the cardiac Na$^+$-K$^+$ pump by ANG II while still retaining the widely accepted role of PKC in Na$^+$-K$^+$ pump regulation.

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