Phorbol Diacetate Potentiates Na⁺-K⁺ ATPase Inhibition by a Putative Endogenous Ligand, Marinobufagenin

Olga V. Fedorova, Natalia A. Dorofeeva, Denis A. Lopatin, Edward G. Lakatta, Alexei Y. Bagrov

Abstract—Several vasoconstrictor agents can regulate the phosphorylation status of the Na⁺-K⁺ ATPase (NKA). We have recently demonstrated that mammalian tissues contain an endogenous bufadienolide, digitalis-like α₁-NKA—selective ligand, marinobufagenin (MBG). Protein kinase C induces phosphorylation of the α₁-NKA isoform, the major isoform in vascular smooth muscle, kidney, and heart cells. We hypothesized that protein kinase C–induced phosphorylation of NKA can potentiate the effect of endogenous digitalis-like ligands, and that such potentiation can occur in an NKA isoform–specific fashion. A protein kinase C activator, phorbol 12,13-diacetate (PDA, 50 nmol/L), induced phosphorylation of the α₁-NKA from human mesenteric artery (HMA) sarcolemma and rat kidney but not that of the α₁-NKA from rat fetal brain. In HMA sarcolemma, which predominantly contains α₁-NKA, PDA (50 nmol/L) potentiated the NKA-inhibitory effect of MBG at the level of high-affinity binding sites (0.05±0.03 nmol/L versus 4.0±1.7 nmol/L, P<0.05). In contrast, PDA did not affect the NKA inhibition by ouabain, an α₁-NKA ligand. In isolated endothelium-denuded HMA artery rings, 50 nmol/L PDA potentiated the MBG-induced vasoconstriction (EC₅₀, 17±6 nmol/L versus 150±40 nmol/L; P<0.01). Our results suggest that α₁-isoform–specific NKA inhibition by the endogenous digitalis-like ligand, MBG, is substantially enhanced via NKA phosphorylation by protein kinase C. Thus, an interaction of protein kinase C–dependent phosphorylation and MBG on NKA activity may underlie the synergistic vasoactive effects of MBG and other endogenous vasoconstrictors in hypertension. (Hypertension. 2002;39:298-302.)

Key Words: drug therapy ■ ouabain ■ Na⁺-K⁺-exchanging ATPase ■ vasoconstriction ■ protein kinases ■ blood pressure ■ bufanolides

Various vasorelaxants and vasoconstrictors can regulate vascular Na⁺-K⁺ ATPase (NKA) activity via its phosphorylation/dephosphorylation by protein kinases and phosphatases.1–3 Protein kinases phosphorylate the sodium pump in a tissue- and isoform-specific fashion.4,5 Protein kinase C (PKC) directly phosphorylates the α₁-NKA isoform.6,7 Several endogenous digitalis-like NKA inhibitors exist in mammalian plasma.8 A cardenolide, a ouabain-like compound, was the first endogenous sodium pump inhibitor to be purified.9 More recently, we have demonstrated that an endogenous bufadienolide NKA inhibitor, marinobufagenin (MBG),10 exhibits greater affinity for the α₁-NKA isoform than for the ouabain-sensitive α₁-isoform.11–13 The α₁-NKA is the major isoform in the kidney, vascular smooth muscle, and adult cardiomyocytes.14,15 Although phosphorylation of NKA by PKC can affect the sensitivity of this enzyme to ouabain,16 it is not known whether PKC phosphorylation of specific NKA isoforms is implicated in NKA inhibition by endogenous digitalis-like inhibitors, such as MBG. We hypothesized that protein kinase C–induced phosphorylation of the NKA can potentiate the effect of endogenous digitalis-like ligands, and that such potentiation can occur in a NKA isoform–specific fashion. The purpose of present study was to determine whether PKC-induced–specific NKA isoform phosphorylation affects NKA inhibition and vasoconstriction in human mesenteric arteries (HMA) by 2 putative endogenous ligands, ouabain and MBG.

Methods

Preparation of Membranes

Experiments were performed on rings of second- or third-order branches of HMA obtained from 42 male patients (50±5 years) undergoing abdominal surgery because of intestinal adenocarcinoma. All subjects gave informed consent to these studies, which had been approved by the local ethics committee. HMA sarcolemma was prepared as described previously in detail using differential membrane centrifugation in the discontinuous sucrose gradients (0.32, 0.8, 1.0, 1.2, and 1.4 mol layers). The band rich in sarcolemma was collected at the 0.8-mol interface.17

Na⁺-K⁺-ATPase

NKA activity was measured as reported previously.17 Membranes were pretreated with alamethicin (0.5 mg/mg protein). Aliquots of

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From the Laboratory of Cardiovascular Science, National Institute on Aging, National Institutes of Health (O.V.F., E.G.L., A.Y.B.), Baltimore, Md; and Laboratory of Pharmacology, Sechenov Institute of Evolutionary Physiology and Biochemistry (N.A.D., D.A.L., A.Y.B.), St Petersburg, Russia. Correspondence to Alexei Bagrov, Laboratory of Cardiovascular Science, Intramural Research Program, National Institute on Aging, 5600 Nathan Shock Dr, Baltimore, MD 21224. E-mail BagrovA@grc.nia.nih.gov
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sarcolemna (100 µL, 1 µg protein/well) were preincubated for 30 minutes at 37°C with compounds as described and then incubated for 1 hour at 37°C in NUNC polystyrene plates in the medium (in mmol/L NaCl 100, KCl 10, MgCl2 3, EDTA 1, Tris 50, ATP 2, and NaH2PO4 0.4, NaHCO3 19, and glucose 5.4 and were gassed with a mixture of 95% O2 and 5% CO2 (pH 7.45). Isometric contractions were recorded.19 The arterial rings were constricted twice with 80 mmol/L KCl, and after 60 minutes, effects of MBG in the absence or presence of PDA were studied.

Western Blotting
Solubilized protein from HMA sarcolemna, rat kidney, and brain were separated by 8% Tris-glycine polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane.18 The proteins were visualized using monoclonal mouse anti-α2-NKA (Upstate Biotechnologies, 1:2000), or anti-α3-NKA (Affinity BioReagents Inc, 1:500) antibody, followed by incubation with peroxidase-conjugated anti-mouse antiserum (Amersham Corp.).

NKA Phosphorylation
NKA phosphorylation was assessed as previously described,7 with some modifications. Membranes were pretreated with alamethicin, and preincubated with or without phorbol 12,13-diacetate (PDA) for 3 minutes at 30°C in a buffer containing 10 mmol/L Tris phosphate, 5 mmol/L magnesium acetate, and 0.5 mmol/L CaCl2. Phosphorylation was initiated by the addition of [γ-32P]ATP (1000 cpm/pmol) to a final concentration of 70 µmol/L. The suspension was incubated for 30 minutes at 30°C, and the reaction was quenched with an equal volume of Novex Tri-Glycine SDS sample buffer. Electrophoresis was performed as above (Western Blotting). Phosphoproteins were visualized by a 12- to 24-hour exposure of nitrocellulose membranes on Kodak XAR-5 film, and density of bands was quantified (Bio-Rad Gel Doc 1000, Bio-Rad Laboratories).

Isolated Mesenteric Artery Contractile Studies
Endothelium-denuded rings of second- or third-order branches of HMA were suspended at a resting tension of 1.0 g in an organ bath and superfused at 37°C with a solution containing (in mmol/L) NaCl 130, KCl 4.0, CaCl2 1.8, MgCl2 1.0, NaH2PO4 0.4, NaHCO3 19, and glucose 5.4 and were gassed with a mixture of 95% O2 and 5% CO2.

Figure 1. A, Western blots of the α1- and α3-isomers of the NKA in HMA sarcolemna (Sarc) (10 µg protein per lane) and rat kidney (Kid) and rat fetal brain (Br) (2 µg protein per lane). B through D, The effects of PDA on phosphorylation of α1-NKA (HMA sarcolemna and rat kidney membranes) or α3-NKA isoforms (rat fetal brain). Upper panels, Representative autoradiograms of 32P-labeled proteins after SDS-gel electrophoresis and transfer of the samples to nitrocellulose. Lower panels, Optical density of the bands; each bar represents mean±SEM from 4 to 6 measurements. *P<0.01 vs control (0 nmol/L PDA), 1-way ANOVA followed by Bonferroni test.

Statistics
Data are presented as mean±SEM. Statistical analyses utilized repeated measures and 1-way ANOVA followed by multiple comparisons tests (GraphPad Prism, GraphPad Software Inc).

Reagents
Chemicals were obtained from RBI International. MBG was purified from the venom of Bufo marinus toad.19

Results
Figure 1A demonstrates the abundance of the α1- and α3-NKA isoforms in sarcolemna from HMA and in 2 reference membrane preparations, microsomes from rat kidney and rat fetal brain. The α1-NKA was abundant in the kidney and in HMA sarcolemna but was practically undetectable in fetal brain synaptosomes. Conversely, α3-NKA immunoreactivity was present in rat fetal brain membranes but was absent in renal membranes and in HMA sarcolemna.

Figure 1B through 1D illustrates the NKA isoform specificity of PDA-induced phosphorylation. PDA, in a concentration-dependent manner (1 to 100 nmol/L) induced phosphorylation of the α1-NKA isoform from HMA sarcolemna (Figure 1B) and rat kidney (Figure 1C). In contrast, PDA did not affect the phosphorylation of NKA from rat fetal brain, which predominantly contains the α3-isoform (Figure 1D).

The effects of PDA on NKA inhibition by ouabain and MBG were compared in HMA sarcolemna (Figure 2A). Both MBG and ouabain inhibited HMA sarcosomal NKA in a concentration-dependent manner, but MBG was far more potent that ouabain. The IC50 of MBG and ouabain in a 1-site competition model was 52±7.5 nmol/L and 1.8±0.4 µmol/L, respectively. Analysis of the concentration-response curves
using a 2-site competition model indicated that both ligands inhibited the NKA activity at the level of higher- and lower-affinity receptor sites. The IC_{50} for each affinity site is listed in the Table. Pretreatment of the membranes with PDA potentiated NKA inhibition by MBG at the level of high (nanomolar) but not low (micromolar) receptor sites (Figure 2B). Conversely, PDA did not significantly affect the ouabain-induced NKA inhibition (Figure 2C).

The effect of PDA (50 nmol/L) on MBG vasoconstriction of isolated HMA rings is illustrated in Figure 3. Pretreatment of the vascular rings with PDA potentiated the effect of MBG. The MBG EC_{50} for vasoconstriction in the absence and in the presence of PDA was 160±30 and 18±7 nmol/L, respectively.

**Discussion**

The main results of the present study are that PDA, a PKC activator, induces the phosphorylation of the sodium pump α_{1}-isoform in HMA, and potentiates the NKA inhibitory and vasoconstrictor effects of MBG, an α_{1}-NKA ligand. NKA isoforms are distributed throughout cardiovascular system in an inhomogeneous and tissue-specific manner. We have shown that the α_{1}-NKA isoform in rat aortae is mainly localized to the sarcolemma, whereas the α_{3}-NKA predominates in the nerve ending membranes. The present results, similar to our previous observations, demonstrate that HMA sarcolemma contains a greater amount of the α_{1}-NKA than of the α_{3}-NKA isoform protein. In HMA sarcolemma, MBG inhibited the NKA more effective than ouabain in the present experiments, in agreement with our previous observations, indicating that MBG exhibits high affinity to the α_{1}-NKA from rat kidney and rat aortic sarcolemma.

Although previous studies have noted that PKC-induced NKA phosphorylation affected both the functional properties of this enzyme and the maximal NKA ouabain binding and sensitivity, it was unknown whether NKA phosphorylation by PKC affects the inhibitory activity of isofom specific endogenous ligands, such as MBG. The present results show that PDA pretreatment, indeed, potentiates both vasoconstricitor and NKA inhibitory effects of MBG.

In the present study, PDA induced the phosphorylation of α_{1}-NKA from 2 tissues, HMA sarcolemma and rat kidney, whereas it did not affect the phosphorylation of NKA from rat fetal brain, in which the α_{3}-NKA is the predominant subunit isoform. These observations are in accord with the recent finding that activation of PKC results in phosphorylation of MBG and Ouabain With the NKA From HMA Sarcolemma

<table>
<thead>
<tr>
<th>Treatment</th>
<th>High-Affinity IC_{50}, nmol/L</th>
<th>Low-Affinity IC_{50}, μmol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>MBG</td>
<td>OUABAIN</td>
<td>MBG</td>
</tr>
<tr>
<td>50 nmol/L PDA (−)</td>
<td>4.0 (1.7)</td>
<td>3.3 (2.1)</td>
</tr>
<tr>
<td>50 nmol/L PDA (+)</td>
<td>0.05 (0.03)</td>
<td>2.7 (1.2)</td>
</tr>
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Values are n (95% confidence limits). The average of 6 to 9 measurements of inhibition of HMA by MBG and ouabain in the presence (+) or absence (−) of PDA at the level of high- and low-affinity binding (2-site competition model).

By repeated measures ANOVA followed by Neuman-Keuls test: high-affinity IC_{50} for MBG in the presence of PDA vs PDA (−), P<0.05.

![Figure 2. The NKA activity of HMA sarcolemma after inhibition by MBG or ouabain. A, MBG and ouabain. B, MBG in the presence or absence of 50 nmol/L PDA (2-site competition model). C, Ouabain in the presence or absence of 50 nmol/L PDA (2-site competition model). Each point represents the mean±SEM of 6 to 9 measurements. Mean values of IC_{50} for the 2-site competition model are listed in the Table. By repeated measures ANOVA followed by Neuman-Keuls test: MBG vs ouabain, P<0.001; MBG vs MBG in the presence of PDA, P<0.01; ouabain vs ouabain in the presence of PDA, P>0.05.](http://hyper.ahajournals.org/)

![Figure 3. The vasoconstrictor effects of MBG in isolated HMA rings in the presence or absence of 50 nmol/L PDA. Each point represents the mean±SEM of 6 to 10 experiments. By repeated measures ANOVA followed by Neuman-Keuls test: MBG vs MBG in the presence of PDA, P<0.01.](http://hyper.ahajournals.org/)
the α7-NKA isoform. Our results show that PKC, in a NKA isoform–specific fashion, modulates the interactions between the sodium pump and its endogenous inhibitory ligands.

The effects of PKC on NKA activity have been controversial. Vasilets et al18 have reported that a mutation of Ser23 of the α7-subunit of rat NKA, which mimics the effect of PKC-induced phosphorylation, was associated with a dramatic decrease in the ouabain sensitivity of the NKA. Conversely, Satoh et al23 observed that pretreatment of isolated rat atria with phorbol diesters potentiates the positive inotropic effect of micromolar concentrations of ouabain. The present results indicate a synergistic interaction of MBG and PKC phosphorylation on NKA inhibition. In HMA sarcolemma, in the presence of PDA, the NKA inhibitory effect of MBG on sodium pump was potentiated at the level of high-affinity sites, i.e., those sites that may be targeted by the nanomolar concentrations of MBG observed in vivo. We have previously demonstrated that substantial increases in the plasma level and/or renal excretion of MBG accompany the blood pressure elevation in several hypertensive states, including Dahl salt-sensitive rats on a high NaCl intake,13 preeclampsia,24 and hypertension in end stage renal disease.25 Therefore, nanomolar or subnanomolar levels of MBG immunoreactivity are observed in vivo in hypertensive states13,24,25 may be sufficient to induce a substantial in vivo inhibition of the sodium pump and to affect the vascular tone. Thus, our present findings show that in the presence of PDA phosphorylation of α7-NKA, 1 nmol/L MBG produces 23% of the maximal vasoconstriction and inhibits the sarcolemmal NKA by 45%. Low concentrations of vasoconstrictors, such as angiotensin II and endothelin-1, have also been reported to activate PKC.26–29 Increased levels of these vasoconstrictors, eg, in the context of hypertension, may potentially enhance vasoconstrictor action of MBG via PKC-sensitive mechanism.

Recently, we observed that cicletanine, an antihypertensive compound that directly inhibits PKC,30 relaxed HMA rings that were precontracted with MBG.24 This vasorelaxant effect of cicletanine did not occur in the presence of PDA. Thus, taken together, our present and previous results suggest that PKC activation potentiates, whereas PKC inhibition reverses both the NKA inhibitory and vasoconstrictor effects of MBG, and that PKC phosphorylation of the α7-subunit of the sodium pump, at least in part, underlies this phenomenon. We propose that PKC and the endogenous α7-NKA–specific isoform ligand, MBG, exhibit interactive effects to inhibit the vascular NKA. Thus both are potential targets for pharmacological intervention of pathologically affected vascular NKA activity.

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