Myocardial PKC β2 and the Sensitivity of Na/K-ATPase to Marinobufagenin Are Reduced by Cicletanine in Dahl Hypertension

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Abstract—Marinobufagenin (MBG), an endogenous ligand of α-1 Na/K-ATPase, becomes elevated and contributes to hypertension in NaCl-loaded Dahl-S rats (DS). Protein kinase C (PKC) phosphorylates α-1 Na/K-ATPase and increases its MBG sensitivity. Cicletanine, an antihypertensive compound with PKC-inhibitory activity, reverses MBG-induced Na/K-ATPase inhibition and vasoconstriction. We hypothesized that increased PKC levels in sodium-loaded hypertensive DS would sensitize α-1 Na/K-ATPase to MBG and that PKC inhibition by cicletanine would produce an opposite effect. We studied the effects of cicletanine on systolic blood pressure, left ventricular PKC isoforms, cardiac α-1 Na/K-ATPase levels, and sensitivity to MBG in hypertensive DS. Seven DS received 50 mg · kg⁻¹ · d⁻¹ cicletanine, and 7 DS received vehicle during 4 weeks of an 8% NaCl diet. Vehicle-treated rats exhibited an increase in blood pressure, left ventricular mass, MBG excretion (74±11 vs 9±1 pmol/24 h, P<0.01), myocardial α-1 Na/K-ATPase protein, and PKC β2 and δ. The sensitivity of Na/K-ATPase to MBG was enhanced at the level of high-affinity binding sites (IC₅₀ 0.8 vs 4.4 nmol/L, P<0.01). Cicletanine-treated rats exhibited a 56-mm Hg reduction in blood pressure (P<0.01) and a 30% reduction in left ventricular weight, whereas cardiac α-1 Na/K-ATPase protein and MBG levels were unchanged. In cicletanine-treated rats, PKC β2 was not increased, the sensitivity of Na/K-ATPase to MBG was decreased (IC₅₀=20 μmol/L), and phorbol diacetate–induced α-1 Na/K-ATPase phosphorylation was reduced versus vehicle-treated rats. In vitro cicletanine treatment of sarcolemma from vehicle-treated rats also desensitized Na/K-ATPase to MBG, indicating that this effect was not solely attributable to a reduction in blood pressure. Thus, PKC-induced phosphorylation of cardiac α-1 Na/K-ATPase is a likely target for cicletanine treatment. (Hypertension. 2003;41:505-511.)

Key Words: rats, Dahl ■ Na/K-transporting ATPase ■ digitalis-like factor ■ bufanolides ■ hypertrophy ■ protein kinases ■ antihypertensive agents

Endogenous digitalis-like sodium pump ligands (SPLs) increase during states of NaCl retention to induce inhibition of the renal tubular sodium pump and natriuresis. Enhanced SPL levels, however, can also inhibit Na/K-ATPase (NKA) in cardiovascular tissues and raise arterial pressure. Recently, we have shown that plasma and urine levels of marinobufagenin (MBG), an endogenous bufadienolide NKA inhibitor, rather than endogenous ouabain, increases and contributes to hypertension in Dahl salt-sensitive rats (DS) maintained on a long-term high-NaCl intake. In vitro, MBG acts as a vasoconstrictor and exhibits selectivity toward α-1 isoform of the sodium pump, ie, the major isoform in renal tubules and adult cardiomyocytes.

Protein kinase C (PKC) can affect NKA activity by altering its phosphorylation state. This modulatory effect of PKC is specific to the NKA α-1 isoform. In arterial sarcolemma, PKC induces the phosphorylation of α-1 NKA and enhances the NKA sensitivity to MBG. Recently, we have demonstrated that cicletanine, a furopyridine antihypertensive compound with natriuretic and vasorelaxant actions, directly inhibits PKC activity and antagonizes the vasoconstrictor and NKA-inhibitory effects of MBG by a PKC-sensitive mechanism. Phorbol diacetate, which stimulates PKC, reversed the effect of cicletanine on MBG-induced NKA inhibition and vasoconstriction.

Thus, it appears that both SPLs and PKC may modulate NKA activity in cardiovascular tissues. Because hypertension is known to be associated with activation of PKC in left ventricular (LV) myocardium, it is possible that enhanced phosphorylation of α-1 NKA could sensitize the LV sodium pumps to MBG. We hypothesized that cicletanine, by dephosphorylation of α-1 NKA, would reduce its sensitivity to MBG in hypertensive DS. In the present experiment, we determined whether changes in myocardial PKC isoforms,
the α-1 NKA amount, or the sensitivity of α-1 NKA to its putative endogenous ligand, MBG, accompany the antihypertensive effect of cicletanine.

### Methods

#### General

The protocol of the study was approved by the Animal Care and Users Committee of the Gerontological Research Center, National Institute on Aging. Twenty-two 5-week-old DS were obtained from Harlan-Sprague-Dawley Inc, Indianapolis, Ind. Animals were adapted at 26°C for 1 week. At the age of 6 weeks, eight DS were humanely killed and served as controls, and 14 were administered an 8% NaCl diet for 4 weeks. Seven of these received cimetidine orally, 50 mg·kg·d, and 7 received vehicle. During the subsequent 28-day experimental period, body weight, systolic blood pressure (SBP), water consumption, and urine output were measured weekly. SBP was recorded by tail-cuff plethysmography. Transthoracic echocardiography was performed in rats anesthetized with 40 mg/kg sodium pentobarbital IP at baseline and during week 4 of the experiment (Hewlett-Packard Sonos 5500 with a 12-MHz pediatric probe). At 28 days, the rats treated with vehicle and cimetidine were anesthetized by an intraperitoneal injection of 60 mg/kg sodium pentobarbital and humanely killed by exsanguination.

#### Myocardial NKA

LV sarcolemma was prepared from pooled LV myocardium homogenates, as described previously, by differential membrane centrifugation in 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. Sarcolemma was permeabilized by pretreatment with alamethicin (0.5 mg/ml protein). Aliquots of sarcolemma (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, KC1 10, MgCl2 3, EDTA 1, Tris 50, ATP 2, and NaN3 5, pH 7.4). The reaction was stopped by addition of a quenching solution (1N sulfuric acid and 0.5% ammonium molybdate), followed by color reaction with 0.02% SnCl4. NKA activity was estimated as the difference between total ATPase activity in the presence and absence of 5 mmol/L ouabain. The NKA activity in LV sarcolemma was 10.9±1.0 μmol Pi·mg−1 protein·h−1 and comprised 40% of the total ATPase activity.

#### Western Blotting of α-1 NKA and PKC Isoforms

Solubilized protein from LV sarcolemma was separated by 8% SDS-PAGE and transferred to nitrocellulose membrane. The proteins were visualized by a monoclonal mouse anti-α-NKA (Upstate Biotechnologies, Lake Placid, NY; 1:2000) and by polyclonal antibodies to PKC α, β, δ, ε, and ζ isoforms (BBI), followed by incubation with peroxidase-conjugated anti-mouse and anti-rabbit antisera (Amersham Corp). Immunoreactivity was detected by enhanced chemiluminescence (Hyperfilm-ECL, Amersham). Optical density of the bands was estimated by using a Bio-Rad Gel Doc 1000 single-wavelength multianalysator and the Multi-Analyser program (Bio-Rad Laboratories).

#### NKA Phosphorylation

NKA phosphorylation was assessed as previously described. Membranes (1 mg protein per ml) from LV sarcolemma were pretreated with alamethicin and preincubated with or without phosphol 12,13-diacylate (PDA, 1 mmol/L) for 3 minutes at 30°C in a buffer containing (in mmol/L) Tris phosphate 10, magnesium acetate 5, and CaCl2 0.5. Phosphorylation was initiated by the addition of [γ-32P]ATP (1800 counts per minute/pmol) to a final concentration of 60 μmol/L. The suspension was incubated for 30 minutes at 30°C and the reaction quenched with an equal volume of Novex Tris-glycine sodium dodecyl sulfate sample buffer. Electrophoresis was performed as described previously (Western blotting) except that 12% Tris-glycine gel was used. Phosphoproteins were visualized by...
12- to 24-hour exposure of the nitrocellulose membranes on Kodak XAR-5 film; density of the bands was quantified as also previously described (Bio-Rad Gel Doc 1000).

Immunoassays

The MBG immunoassay, based on competition between immobilized antigen (MBG-glycoside-RNAase) and SPLs within the sample for a limited amount of binding sites on polyclonal rabbit MBG (raised against MBG-glycoside-BSA) antibody, was performed as recently described.4 Urinary cGMP was measured by using a Biotrak enzyme immunoassay (Amersham Pharmacia Biotech).

Statistics

Results are expressed as mean±SEM and were analyzed statistically by a 1-way or repeated-measures ANOVA followed by a Bonferroni test, or by a 2-tailed t-test (when appropriate) (GraphStat Prism, GraphStat Inc).

Chemicals

Chemicals were obtained from Sigma-Aldrich Chemical Co unless otherwise indicated. Cicletanine was a gift from the IPSEN Institute (Paris, France).

Results

Changes in body weight and water and NaCl intake over the course of the experiment were similar in both experimental groups (Tables 1 and 2). As illustrated in Figure 1A, rats on a high-NaCl diet exhibited a substantial increase in SBP, which was reduced in NaCl-loaded, cicletanine-treated rats. The plasma sodium concentration was the same in cicletanine-treated rats as in vehicle-treated rats. However, compared with vehicle-treated rats, cicletanine-treated rats exhibited less diuresis, a greater glomerular filtration rate, greater sodium excretion, and a higher hematocrit (Table 1).

The development of hypertension during high-NaCl intake in vehicle-treated rats was associated with a significant increase in plasma levels and renal excretion of MBG, whereas excretion of cGMP decreased (Figure 1B and 1C). No difference in MBG and cGMP excretion between the cicletanine-treated and vehicle-treated DS was observed. The high-NaCl diet in vehicle-treated DS was accompanied by LV hypertrophy and reduced LV pump function (Table 2). LV hypertrophy did not occur in cicletanine-treated animals, and their LV function was less impaired than in vehicle-treated rats.

Four weeks of high-NaCl intake in vehicle-treated rats resulted in a 37% decrease in NKA activity (Table 3) and a

| TABLE 3. Effect of In Vivo and In Vitro Cicletanine Treatment on the Activity of LV Sarcolemmal NKA and Its Sensitivity to MBG at the Level of Higher- and Lower-Affinity Binding Sites (2-Site Competition Model) |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|
|                                | Baseline (Low NaCl) | 4 Weeks of High NaCl Intake |
|                                | No Treatment | Cicletanine In Vitro | Vehicle | Cicletanine In Vivo | Cicletanine In Vitro |
| NKA activity, μmol Pi/mgprot/h | 10.9±1.0  | 11.6±0.7 | 6.9±0.2* | 9.8±0.5* | 7.4±0.1 |
| \( IC_{50} \) at the level of high affinity sites, nmol/L | 4.4±1.3 | Not detectable* | 0.8±0.2 | Not detectable* | Not detectable* |
| \( IC_{50} \) at the level of low affinity sites, μmol/L | 37±11 | 35±12 | 53±12 | 20±2.4 | 5.6±1.4 |

Data are mean±SEM of 5–8 inhibitory curves. For NKA activity: 2-tailed t test; for individual analysis of concentration-effect curves for MBG: nonlinear regression using 1- and 2-site competition models; for comparisons of the NKA inhibition curves: repeated measures ANOVA followed by Newman-Keuls test (intergroup comparisons).

* \( P<0.01 \) vs baseline (no treatment); \( \# P<0.01 \) vs vehicle treated DS on a high NaCl intake.
45% increase in the \(\alpha-1\) NKA isoform protein in LV sarcolemma (Figure 2A). The NKA activity in cicletanine-treated DS was higher than that in vehicle-treated rats, but the levels of myocardial \(\alpha-1\) NKA protein did not differ between the 2 groups.

In LV sarcolemma from control rats, MBG exhibited NKA inhibition, which occurred at both the level of higher- (nanomolar) and lower- (micromolar) affinity receptor sites (Figure 2B and Table 3). The development of LV hypertrophy in vehicle-treated DS on a high-NaCl diet was associated with a significant increase in the MBG sensitivity of NKA high-affinity sites from LV sarcolemma. In contrast, the NKA from cicletanine-treated DS was much less sensitive to MBG than that from vehicle-treated rats.

As presented in Figure 3, baseline levels of phosphorylation of \(\alpha-1\) NKA did not vary between cicletanine-treated and vehicle-treated groups. PDA (1 nmol/L) significantly enhanced the levels of \(\alpha-1\) NKA phosphorylation in the membranes prepared from vehicle-treated DS but did not affect the membranes prepared from cicletanine-treated animals.

To demonstrate that the aforementioned effects of cicletanine were not solely attributable to a reduction in BP, we studied the effect of in vitro cicletanine treatment on MBG-induced NKA inhibition in LV sarcolemma from control DS maintained on a 0.2% NaCl intake and from vehicle-treated rats on an 8% NaCl intake. The in vitro pretreatment of sarcolemma from control DS with 100 \(\mu\)mol/L cicletanine did not affect the NKA activity but did induce a rightward shift of the concentration-effect curve of MBG (Table 3). No high-affinity component in the effect of MBG was observed after this treatment (Figure 4A). In sarcolemma from hypertrophied hearts, in vitro cicletanine treatment reduced the MBG sensitivity of NKA, but antagonism of the MBG-induced NKA inhibition was greater than that observed in the control hearts (Figure 4B and Table 3).

The sarcolemma-bound PKC isoform composition was altered in the hypertrophied LV of vehicle-treated rats on a high-NaCl diet. Compared with control DS, LV sarcolemmal PKC \(\beta2\) and \(\delta\) increased, PKC \(\zeta\) decreased, and PKC \(\alpha\) and \(\epsilon\) were unchanged (Figure 5). In cicletanine-treated rats, membrane levels of PKC \(\beta2\) and \(\delta\) were reduced versus those in vehicle-treated animals and were not significantly different from baseline. PKC \(\zeta\) levels were reduced by a high-salt diet versus baseline, with or without cicletanine treatment.

**Discussion**

The present results demonstrate that during the development of LV hypertrophy in DS on a high-NaCl intake, (1) levels of an endogenous ligand of \(\alpha-1\) NKA, MBG, are enhanced; (2)
the LV α-1 NKA protein concentration increases; and (3) the sensitivity of LV NKA to MBG is also increased. These changes were accompanied by increases in the levels of LV sarcolemmal β2 and δ PKC. Cicletanine treatment substantially reduced the BP increase in response to a high-NaCl diet and prevented the increase in the sensitivity of myocardial NKA to MBG, despite the continued presence of increased MBG production and enhanced levels of LV α-1 NKA protein. Furthermore, sarcolemmal PKC β2 and δ levels in cicletanine-treated DS did not significantly differ from those in control animals, and PDA-induced α-1 NKA phosphorylation was much less than in the vehicle-treated group.

In the present experiment, cicletanine administration reduced SBP by >50 mm Hg, as did comparable doses of cicletanine in hypertensive rats in several previous studies.\(^{16,17}\) Previously, the antihypertensive effects of cicletanine have been attributed to several mechanisms but primarily to the inhibition of cGMP-dependent phosphodiesterase.\(^{18,19}\) If this were the case, it might be expected that cicletanine would increase the plasma levels and urinary excretion of cGMP. In the present study, cicletanine treatment did not affect cGMP excretion, which reflects plasma cGMP levels.\(^{20}\) Thus, in DS at least, cGMP-dependent phosphodiesterase is unlikely a major target of cicletanine. Previously, cicletanine was observed to be especially effective in reducing the BP in DS on a high-NaCl intake.\(^{21}\) Our present observations suggest that in this context, cicletanine exhibits functional antagonism of the NKA inhibitory effect of elevated levels of MBG in LV myocardium. Whether or not cicletanine exerts its in vivo direct vascular effect owing to the same mechanism in DS remains to be proved. Our recent results, indeed, demonstrate that the vasodilatory effect of cicletanine is PKC dependent in human mesenteric arteries precontracted with MBG.\(^{11}\)

In the present study, cicletanine treatment of DS was associated with an improvement of renal function. Previous studies also documented that cicletanine, even at subpressor doses, exhibited renal-protective effects.\(^{22}\) The relevance of NKA- and PKC-dependent mechanisms to the renal action of cicletanine remains to be elucidated.

The development of LV hypertrophy in DS caused by the high-NaCl diet was associated with reduced myocardial NKA activity, an increase in sensitivity of the LV NKA to MBG, and an increase in α-1 NKA protein in LV sarcolemma. Concurrently, renal MBG excretion increased 7-fold. MBG induces inhibition of the high-affinity sarcolemmal NKA receptor sites at nanomolar concentrations. Thus, MBG, in nanomolar concentrations that occur in vivo,\(^{4,5}\) is likely to inhibit the high-affinity myocardial NKA receptors. Our findings are consistent with a previous observation of reduced myocardial NKA activity in DS on a high-NaCl intake.\(^{23}\) One previous study demonstrated that development of LV hypertrophy in diabetic rats was associated with increased α-1 NKA abundance in LV sarcolemma.\(^{24}\) whereas expression of α-1 NKA in hypertrophied hearts from rats with deoxycorticosterone-salt hypertension did not change.\(^{25,26}\)

The present results for the first time show that an increase in the α-1 NKA protein level is associated with the development of hypersensitivity of myocardial sodium pumps to an intrinsic α-1 NKA ligand, MBG. DS on a high-NaCl intake develop compensatory LV hypertrophy, which is followed by a transition to congestive heart failure.\(^{27}\) Inhibition of cardiac NKA has a direct influence on myocardial contractility.\(^{28,29}\) Furthermore, recent data indicate that SPLs, including bufadienolides, exhibit direct growth-promoting effects.\(^{30,31}\) Therefore, sensitization of LV NKA to a putative endogenous inotrope, MBG, may be a previously undescribed factor in the pathogenesis of hypertensive heart disease.

In our study, development of hypertension and LV hypertrophy in NaCl-loaded DS was associated with an increase in two PKC isoforms in sarcolemmal membranes, PKC β2 and δ. Translocation of these two PKC isoforms from the soluble to the particulate fraction has previously been implicated in the hypertrophic signaling that underlies LV remodeling in hypertensives.\(^{12,13}\) PKC induces regulatory phosphorylation of the α-1 NKA isoform\(^{4}\) and increases its MBG sensitivity.\(^{10}\) Inhibition of PKC would be expected to exhibit an opposite effect, ie, to antagonize the vasoconstrictor and NKA-inhibitory action of MBG. Recently, we have demonstrated that, indeed, cicletanine does reverse the MBG-induced vasoconstriction and NKA inhibition by way of a PKC-sensitive mechanism.\(^{11}\) This effect of cicletanine was blocked by phorbol ester and was, therefore, modulated by phorbol-sensitive PKC isoforms, such as β2 and δ. Our present results show that although in vivo cicletanine treatment did not affect the increased expression of α-1 NKA protein in the myocardium effected by the high-NaCl diet, it reduced the sensitivity of LV sarcolemmal NKA to MBG and prevented the reduction in sarcolemmal NKA activity. Therefore, the reduced
sensitivity of α-1 NKA to its endogenous ligand, MBG, may be attributable to an effect of cicletanine on a mechanism that modulates the sensitivity of the NKA to SPLs. Because the increase in sarcolemmal PKC β2 and δ that accompanied the increased sensitivity of NKA to MBG in vehicle-treated, NaCl-loaded rats did not occur in cicletanine-treated DS, we hypothesize that this mechanism involves an increase in sarcolemmal PKC β2 and δ contents. Previously, it has been demonstrated that development of LV hypertrophy in hypertensive rats is associated with downregulation of α-2 NKA in the LV myocardium. A decrease in the level of ouabain-sensitive NKA and a resultant increase in the relative abundance of MBG-sensitive α-1 NKA could represent another possible mechanism of sensitization of myocardial sodium pumps to MBG.

It might be argued that the LV myocardium from cicletanine-treated DS was less sensitive to MBG and that the reduced content of sarcolemmal PKC β2 and δ was mainly due to a reduction in the SBP by cicletanine. However, the results of experiments in which in vitro cicletanine treatment of sarcolemma from DS on both lower- and higher-NaCl intake also resulted in a decrease in the MBG sensitivity of LV NKA argue against such a possibility. The cicletanine treatment in vivo reduced the MBG sensitivity of LV NKA to a greater extent than did in vitro cicletanine treatment of membranes from hypertrophied LV. This difference is likely to reflect that fact that, whereas in vitro treatment could produce only inhibition of the PKC, in vivo cicletanine administration also affected its membrane levels. We interpret the reduction in MBG action on the heart by cicletanine, therefore, to result at least in part from its direct effect on these PKC isoforms. This effect may be particularly relevant to other scenarios in which NKA is inhibited by SPLs. Therefore, the interaction of MBG and PKC on the myocardial sodium pump, ie, PKC-induced sensitization of cardiac α-1 NKA to MBG by way of its phosphorylation, would appear to be a likely target for cicletanine.

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References


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