Cicletanine reverses vasoconstriction induced by the endogenous sodium pump ligand, marinobufagenin, via a protein kinase C dependent mechanism

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Rationale **Cicletanine (CIC), an anti-hypertensive compound with direct vascular and natriuretic actions, is especially effective in salt-sensitive hypertension, in which dysregulation of the sodium pump plays an important pathogenic role, and digitalis-like cardiotonic steroids contribute to increased vascular tone. The purpose of the present study was to investigate whether, and by what mechanisms, cicletanine antagonizes the vasoconstrictor effects of cardiotonic steroids in isolated human arteries.**

Methods **The effects of cicletanine on vascular tone were studied in isolated, endothelium-denuded rings of 2nd–3rd-order branches of human mesenteric arteries pre-contracted with bufodienolide marinobufagenin (MBG), an Na/K-ATPase inhibitor, or endothelin-1 (ET-1). Na/K-ATPase activity was measured in sarcolemmal membranes from the mesenteric artery. Activity of rat brain protein kinase C (PKC) was measured using the PepTag phosphorylation assay.**

Results **MBG and ET-1 both induced sustained vasoconstriction in human mesenteric artery rings, and cicletanine relaxed rings pre-contracted with either MBG (EC_{50} = 11 ± 2 μmol/l) or ET-1 (EC_{50} = 6.4 ± 1.1 μmol/l). Although 8-Br-cGMP (100 μmol/l) caused complete vasorelaxation of arterial rings pre-contracted with ET-1, it did not affect the MBG-induced vasoconstriction. An activator of PKC, phorbol diacetate (PDA) (50 nmol/l), attenuated CIC-induced vasorelaxation of mesenteric artery rings pre-contracted with MBG (EC_{50} > 100 μmol/l), but not rings pre-contracted with ET-1 (EC_{50} = 6.5 ± 1.2 μmol/l). In mesenteric artery sarcolemma, 100 nmol/l MBG inhibited the Na/K-ATPase by 68 ± 5% and cicletanine (100 μmol/l) attenuated this Na/K-ATPase inhibition by 85 ± 6%. In the PepTag PKC assay, cicletanine produced a concentration-dependent inhibition of rat brain PKC activity (IC_{50} 45 ± 11 μmol/l). In the presence of 50 nmol/l PDA, 100 μmol/l cicletanine did not antagonize the Na/K-ATPase inhibition by MBG, and did not inhibit the PKC from rat brain.**

Conclusions **Cicletanine antagonizes vasoconstriction induced by Na/K-ATPase inhibition via a PKC-dependent mechanism that does not involve inhibition of cyclic GMP phosphodiesterase (cGMP-PDE). This mechanism of action may be relevant to the greater potency of cicletanine in salt-sensitive hypertension in which plasma levels of endogenous digitalis-like cardiotonic steroids are elevated. Our findings also suggest that PKC is an important factor for cardiotonic steroid–Na/K-ATPase interactions on the vascular tone, and is therefore a potential target for therapeutic intervention in hypertension. *J Hypertens* 2000, 18:209–215 © Lippincott Williams & Wilkins.**

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Introduction

Cicletanine is a furopyridine anti-hypertensive compound with direct vasorelaxant and natriuretic properties [1–3]. The mechanism of natriuretic effects of cicletanine is attributable to the inhibitory action of its sulphoconjugated (+)-enantiomer on the apical Na-dependent Cl/HCO_3 anion exchanger in the cortical diluting segment of the nephron [3]. The nature of the vasorelaxant activity of cicletanine is less well understood but is believed to be due to several mechanisms, including histamine antagonism [4], activation of prostacyclin production [5] and inhibition of low-K_m cyclic GMP phosphodiesterase (cGMP-PDE) [6]. In salt-sensitive hypertension [7], including that occurring in Dahl salt-sensitive rats on a high NaCl intake [8,9], cicletanine is an especially effective vasorelaxant.

Several digitalis-like cardiotonic steroids have been

described in mammalian tissues, including an ouabain-like compound [10], and a bufodienolide, marinobufagenin immunoreactive factor (MBG) [11,12]. Unlike ouabain, MBG exhibits a greater affinity to the α-1 subunit of Na/K-ATPase [13], the main Na pump isoform in renal tubules and vascular sarcolemma [14]. Plasma MBG immunoreactivity, rather than an ouabain-like immunoreactivity, becomes increased in several volume-expanded hypertensive states, such as adrenocorticotrophin-induced hypertension [15], NaCl-induced hypertension in Dahl salt-sensitive rats [16], pre-eclampsia [17] and hypertension in patients with end-stage renal disease [18].

The above considerations – vasoconstrictor activity of MBG; its affinity to the vascular Na pump; evidence for a role of MBG in volume expanded hypertension; and exaggerated efficacy of cicletanine in NaCl-sensitive hypertension – provide the rationale for investigation of the ability of cicletanine to antagonize the effects of MBG. We hypothesized that cicletanine antagonizes MBG vasoconstriction via inhibition of cGMP-PDE. Thus, we compared mechanisms of cicletanine vasorelaxation in vessels pre-contracted with MBG and endothelin-1 (ET-1) in isolated human mesenteric arteries. However, our results demonstrate that although inhibition of cGMP-PDE plays an important role in cicletanine relaxation of ET-1 vasoconstriction, the cicletanine reversal of MBG-induced vasoconstriction and Na/K-ATPase inhibition occurs not via a cGMP-PDE activation but via a protein Kinase C (PKC)-sensitive mechanism. This specific mechanism of cicletanine may render it an effective therapeutic agent in NaCl-sensitive hypertension, in which the plasma volume is expanded and endogenous ligands of the sodium pump are stimulated.

Methods

Isolated mesenteric artery contractile studies

Tissues were obtained from 52 male patients (50 ± 5 years) undergoing abdominal surgery due to intestinal adenocarcinoma; none received radiation therapy or chemotherapy prior to surgery. The 2nd–3rd-order branches of the mesenteric artery were dissected from the tissue, which was not affected by malignant growth. Vascular rings (2.5–4.0 mm diameter) were suspended at a resting tension of 1.0 g in a 10.0 ml organ bath superfused with a medium containing (in mmol/l): NaCl 130, KCl 4.0, $CaCl_2$ 1.8, $MgCl_2$ 1.0, NaH_2PO_4 0.4, $NaHCO_3$ 19, glucose 5.4, at 37°C, and gassed with a mixture of 95% O_2 and 5% CO_2 (pH 7.45). After 60 min equilibration, the arterial rings were contracted twice with 80 mmol/l potassium, and after 60 min, concentration–response curves of vasoconstrictor effects of MBG and ET-1 were determined. To investigate the vasorelaxant ability of cicletanine, arterial rings pre-contracted with 1 μmol/l ET-1 or 100 nmol/l MBG were exposed to increasing concentrations of cicletanine in the presence and absence of the other compounds studied. The percentage relaxation was calculated relative to the plateau of tonic contractile force that was achieved in response to MBG or ET-1. Dose–response curves for vasoconstriction and vasorelaxation were implemented ($n = 6–10$) and EC_{50} values were calculated by linear regression analysis of points producing 20–80% vasoconstriction or vasorelaxation.

Na/K-ATPase from the mesenteric artery

Membranes from mesenteric arteries of a subset of tissues from 12 patients were purified as described previously [19]: 2–3 cm vascular segments were repeatedly washed with a solution containing (in mmol/l): NaCl 130, KCl 5.4; $CaCl_2$ 1.8, $MgCl_2$ 1, glucose 5.4, KH_2PO_4 1.1, $NaHCO_3$ 24, pH 7.4 at 4°C and then cut into 1–2 mm rings. The rings were placed into flasks containing (in mmol/l): sucrose 250, histidine 30, imidazole 5, EDTA 1 (4°C; pH 7.4), minced by scissors and processed with a Polytron 20S homogenizer (Kinematica, Switzerland). The tissue was further homogenized in a glass homogenizer (Glas-Col, Terre Haute, Indiana, USA), and then centrifuged (Sorvall RC-5B, Du Pont Instruments) at 6000 *g*, 15 min, 4°C. The pellet was homogenized in a glass–Teflon homogenizer and added to the supernatant. The combined supernatant was respun at 20 000 *g* for 30 min at 4°C and the resultant supernatant centrifuged (Beckman L8-N, 148 000 *g*, 90 min, 4°C). The sarcolemma was purified as previously reported [19]. The resultant pellet was suspended in a homogenizing medium, applied to discontinuous sucrose gradients consisting of 0.32–1.4 mol layers buffered with 30 mmol/l histidine and 5 mmol/l imidazole (pH 7.4 at 4°C), and centrifuged at 148 000 *g* for 90 min (Beckman L8-N SW28, 4°C). The band at 1.0 mol was aspirated and centrifuged at 148 000 *g* for 90 min, and the pellet was resuspended in 1 ml of homogenizing medium and stored in liquid nitrogen.

Na/K-ATPase activity was measured as reported previously [19]. Aliquots of sarcolemmal suspensions (100 μl containing 1 μg protein/well) were pre-incubated with the compounds studied for 30 min at 37°C, and then incubated for 1 h at 37°C in 96-well polystyrene sample plates (Wallac Oy, Turku, Finland) in assay medium containing (mmol/l): Na 100, K 10, $MgCl_2$ 3, EDTA 1, Tris 50, ATP 2, NaN_3 5 (pH 7.4 at 37°C). The reaction was stopped by the addition of 0.1 ml of quenching solution (1 N sulphuric acid, 0.5% ammonium molybdate), followed by the addition of 0.02% $SnCl_2$. Total ATPase activity was measured by the production of inorganic phosphate (P_i), and Na/K-ATPase activity was estimated as the difference between total ATPase activity in the presence and in the

absence of 1 mmol/l ouabain. The activity of Na/K-ATPase was calculated as μmol of P_i produced per mg protein per hour, and expressed as percentage of residual (uninhibited by ouabain or MBG) activity of Na/K-ATPase. The amount of P_i in the sample was determined at 660 nm for up to 30 min using a Vmax microplate reader (Molecular Devices Inc.). The baseline activity of Na/K-ATPase in the sarcolemma from mesenteric arteries was 5.85 ± 0.24 μmol P_i/mg protein per h. Mg-ATPase and Na/K-ATPase comprised 77 and 23% of the total ATPase activity, respectively.

PKC assay

PKC activity was measured using the PepTag Protein Kinase Assay (Promega, Madison, Wisconsin, USA). The assay is based on the highly specific phosphorylation of a fluorescent PepTag C1 peptide substrate by PKC. PKC purified from rat brain was diluted to 2.5 μg/ml in 100 μg/ml BSA and 0.05% Triton X-100, and was pre-incubated for 30 min at 37°C in the presence or in the absence of cicletanine. Then, 25 ng of PKC, 2 μg of PepTag, a PKC substrate, and a PKC activating solution (phosphatidyl serine, 5 μg) were incubated for 30 min at 30°C in 25 μl of a buffer containing (in mmol/l): HEPES 100, $CaCl_2$ 6.5, DTT 5, $MgCl_2$ 50, ATP 5, pH 7.4. The reaction was stopped by boiling the assay medium in a water bath for 10 min. The samples were further electrophoresed on an 0.8% agarose horizontal gel at 100 V for 15 min, which induced migration of the phosphorylated peptide toward the anode, while non-phosporylated peptide migrated towards the cathode. The ratio of phosphorylated to non-phosphorylated peptide was quantified using a densitometer (BioRad Gel Doc 1000 Darkroom, Hercules, California, USA).

Statistics

The results are expressed as mean ± SEM. The effects of drugs were compared using repeated-measures ANOVA (GraphPad Instat and GraphPad Prism, GraphPad Software Inc., San Diego, California, USA) followed by a multiple comparisons test (Neuman–Keuls) or by a two-tailed *t* test, when appropriate.

Miscellaneous

Chemicals were obtained from RBI International (Natick, Massachusetts, USA). Marinobufagenin (99.5% purity) was purified from the venom of the *Bufo marinus* toad as reported previously [20]. Cicletanine (99.5% purity) was provided by Beaufour-Ipsen Group (Paris, France).

Results

Concentration–response curves of vasoconstrictor effects of MBG and ET-1 in isolated, endothelium-denuded rings of mesenteric artery are given in Figure 1a. Representative recordings of ET-1 and MBG-induced contractile responses are presented in Figures 1b,c. MBG produced a concentration-dependent increase in tension ($EC_{50} = 85 \pm 14$ nmol/l). The MBG-induced contractions developed relatively slowly, reaching a plateau after 40–60 min after addition of the compound to the incubation medium (Fig. 1c), and were resistant to the washout for up to 60 min. By contrast, the contractile responses of arterial rings to ET-1 developed rapidly, reaching a plateau within 10 min ($EC_{50} = 14 \pm 1.6$ nmol/l) (Fig. 1b), and were reversed with washout of the drug.

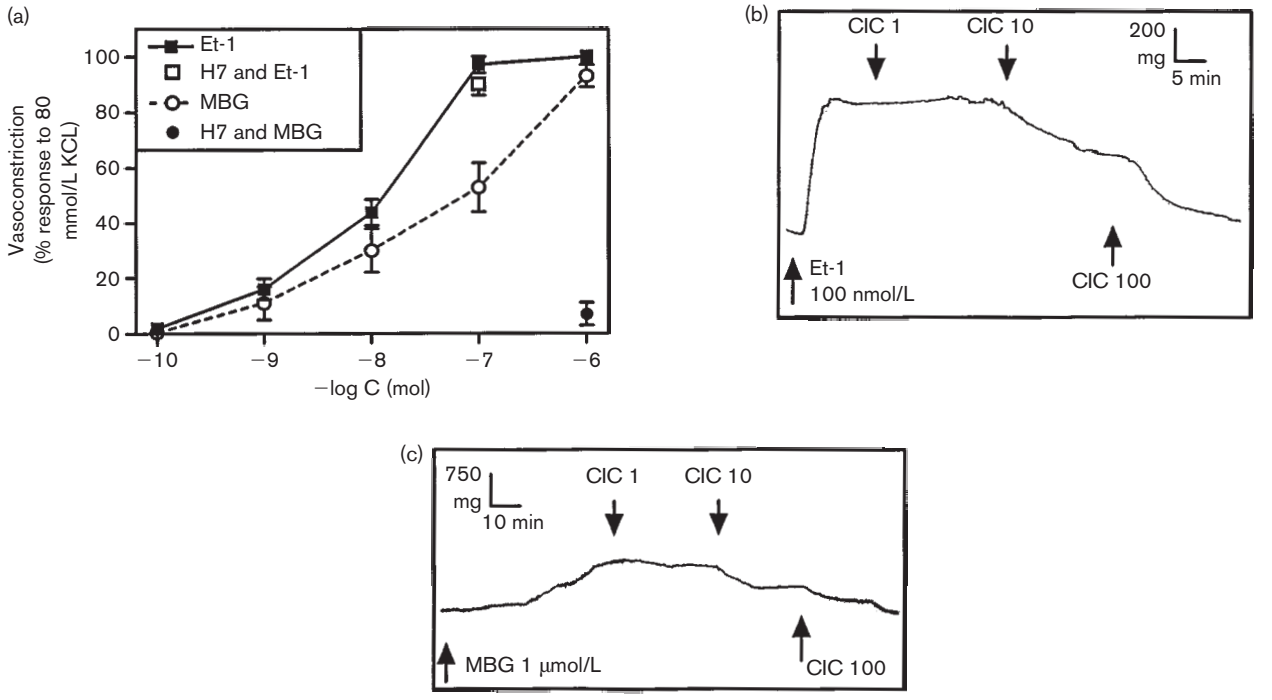
Figure 2a demonstrates that 8-bromo-cGMP-Na, which mimics the effects of guanylate cyclase activators, relaxed the vascular rings pre-contracted with 100 nmol/l ET-1 ($EC_{50} = 8.6 \pm 1.7$ μmol/l). However, even at concentration as high as 500 μmol/l, 8-bromo-cGMP-Na could not relax the mesenteric artery rings pre-contracted with 1 μmol/l MBG.

Cicletanine (1–100 μmol/l) produced a concentration-dependent relaxation of mesenteric artery rings pre-contracted with either 1 μmol/l MBG and with 100 nmol/l ET-1 (Fig. 2b,c). With 1 μmol/l MBG, the EC_{50} was 11 ± 2 μmol/l, and with 100 nmol/l ET-1 it was 6.4 ± 1.1 μmol/l. The kinetics of cicletanine-induced relaxation were similar in the vessels pre-contracted with either MBG and ET-1 (Fig. 1b,c).

Next, the ability of cicletanine to reverse the MBG-induced or ET-1-induced vascular contractions was compared in the absence and in the presence of a PKC activator, PDA. As illustrated in Figures 2b,c, PDA (50 nmol/l) attenuated the cicletanine-induced relaxation of arterial rings pre-contracted with MBG ($EC_{50} > 100$ μmol/l, $P < 0.05$ versus effects of cicletanine alone), but did not affect the cicletanine-induced relaxation of the rings pre-contracted with ET-1 ($EC_{50} = 6.5 \pm 1.2$ μmol/l). Pre-treatment of mesenteric with a PKC inhibitor, H7 (1 μmol/l) did not affect the force of contractions induced by 100 nmol/l ET-1, but blocked vasoconstrictor responses to 1 μmol/l MBG (Fig. 1a).

The baseline activity of Na/K-ATPase in sarcolemma from human mesenteric artery was 5.8 ± 0.2 μmol P_i/mg per h. As shown in Figure 3, the residual Na/K-ATPase activity in the mesenteric artery sarcolemma after treatment with 100 nmol/l MBG was $32 \pm 7\%$ of the baseline value. After treatment with 100 μmol/l cicletanine alone, the residual sarcolemmal Na/K-ATPase activity comprised $85 \pm 4\%$ of the baseline ($P < 0.05$). In the presence of 100 μmol/l cicletanine, 100 nmol/l MBG inhibited the Na/K-ATPase by only 18% (residual Na/K-ATPase activity $82 \pm 9\%$). PDA alone did not affect the Na/K-ATPase activity ($94 \pm 7\%$ of baseline activity). In the presence of 50 nmol/l of the PKC activator, PDA, however, cicletanine did not prevent inhibition of Na/K-ATPase by MBG.

Fig. 1



(a) Vasoconstrictor effects of marinobufagenin (MBG) (○) and endothelin-1 (ET-1) (□) in isolated human mesenteric artery (HMA) rings. (●) Effect of 1 μmol/l MBG in the presence of 1 μmol/l H7. (■) Effect of 100 nmol/l ET-1 in the presence of 1 μmol/l H7. Each point represents means ± SEM from 6–12 experiments. (b) Representative recordings of the vasoconstrictor response to 100 nmol/l ET-1 and of the vasorelaxant effect of cicletanine in isolated HMA. (c) Representative recordings of the vasoconstrictor response to 1 μmol/l MBG and the vasorelaxant effect of cicletanine in isolated HMA. CIC 1, CIC 10 and CIC 100, vasorelaxant effects of 1, 10 and 100 μmol/l cicletanine, respectively.

As shown in Figure 4a, cicletanine (10–100 μmol/l) inhibited the activity of PKC purified from rat brain in a concentration-dependent manner ($IC_{50} = 45 \pm 11$ μmol/l). In the presence of 50 nmol/l PDA, cicletanine at concentration of 100 μmol/l failed to inhibit rat brain PKC (Fig. 4b).

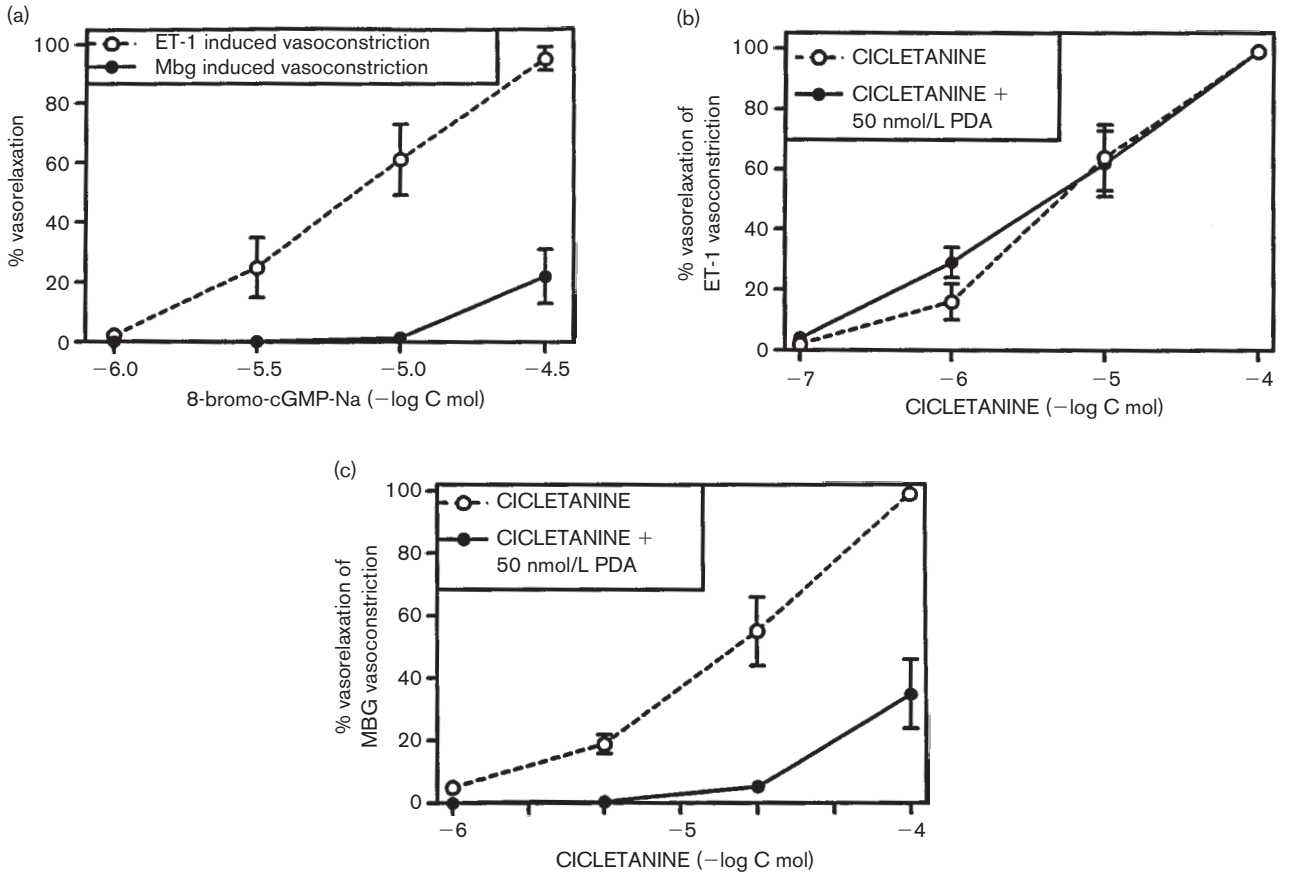
Discussion

The main new finding of the present study is that cicletanine antagonizes vasoconstriction induced by a bufodienolide Na/K-ATPase inhibitor, MBG, in isolated human mesenteric artery rings via a PKC-sensitive mechanism. Previous studies have demonstrated that cicletanine vasorelaxation has multiple mechanisms including antagonism of histamine, stimulation of prostacyclin, and inhibition of low-K_m cGMP-PDE [4–6]. The latter has been attributed to the ability of cicletanine to antagonize vasoconstrictor effects of several pressor agents, including catecholamines, angiotensin II and vasopressin, as well as to a cicletanine-mediated potentiation of the effects of guanylate activators, such as atrial natriuretic peptide (ANP) and sodium nitroprusside [6,2,21,22].

In the present study, cicletanine reversed the MBG-induced contractile responses of arterial rings with approximately the same potency as it antagonized the effects of ET-1, and in previous reports, reversed the effects of noradrenaline and angiotensin II [1,2,21,22]. In the present study, a soluble cGMP analogue, 8-bromo-cGMP-Na, which penetrates the cell membrane and mimics the effects of a guanylate cyclase activator, reversed the ET-1-induced vasoconstriction. This suggests that inhibition of low-K_m cGMP-PDE is involved in reversal of the ET-1-induced vasoconstriction by cicletanine. By contrast, 8-bromo-cGMP-Na failed to relax the vessels pre-contracted by MBG. Therefore, the ability of cicletanine to reverse the MBG-induced vasoconstriction is unlikely to be due to inhibition of the phosphodiesterase.

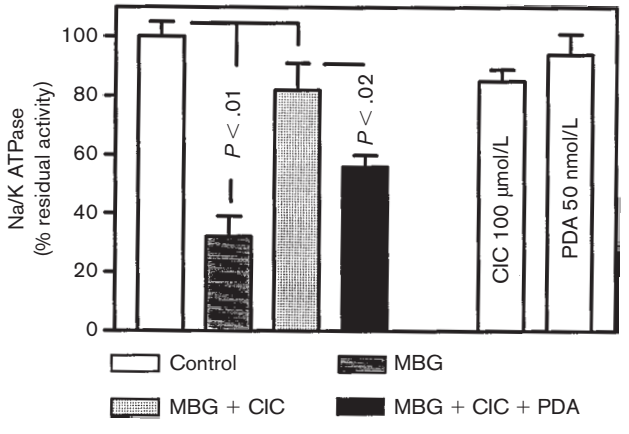
Pre-treatment of vascular rings with a PKC activator, PDA, attenuated the ability of cicletanine to relax rings pre-contracted with MBG, but not with ET-1. This is consistent with our observation that, although pre-treatment of mesenteric artery rings with a PKC inhibitor, H7, blocked the MBG-induced contractile responses, H7 did not reduce the force of ET-1-induced contractions (Fig. 1a). Previously, ET-1 was shown to stimulate PKC in cardiovascular tissues [23].

Fig. 2



(a) Vasorelaxant effect of 8-bromo-cGMP-Na in HMA rings pre-contracted with 1 μmol/l marinobufagenin (MBG) (●) or with 100 nmol/l endothelin-1 (ET-1) (○). (b) Vasorelaxant effect of cicletanine in HMA rings pre-contracted with 100 nmol/l ET-1 in the absence (○) and in the presence (●) of 50 nmol/l PDA. (c) Vasorelaxant effect of cicletanine in HMA rings pre-contracted with 1 μmol/l MBG in the absence (○) and in the presence (●) of 50 nmol/l PDA. Each point represents means ± SEM from six to nine experiments.

Fig. 3

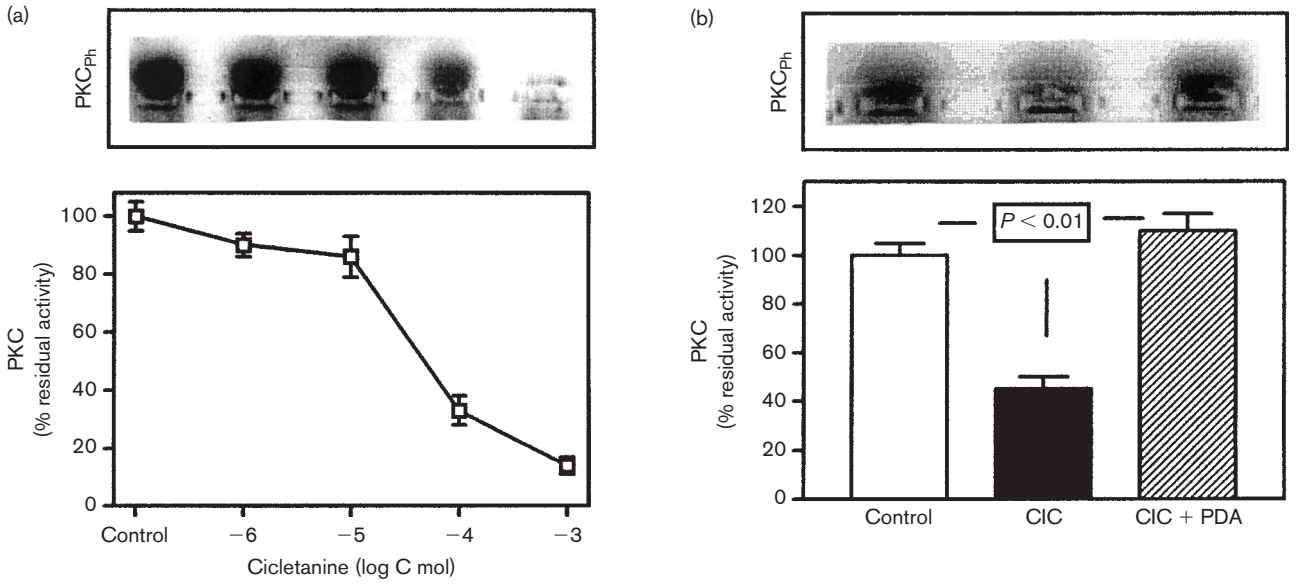


Effects of marinobufagenin (MBG) (100 nmol/l), cicletanine (CIC, 100 μmol/l), phorbol diacetate (PDA) (50 nmol/l) and their combinations on the activity of Na/K-ATPase from HMA sarcolemma. Each bar represents means ± SEM from five to six experiments.

However, the importance of PKC signaling in ET-1-induced contractile responses varies with the type of blood vessels and species studied. For example, in rat basilar and middle cerebral arteries, pre-treatment with H7 only partially attenuates ET-1 contractile responses [24]. In contrast, H7 completely blocks ET-1 vasoconstriction in isolated rat aortae [25]. In rabbit pulmonary vein pre-contracted with ET-1, H7 induced vasorelaxation, while pre-treatment of the tissue with H7 did not affect the contraction induced with ET-1 [26].

In the present study, cicletanine inhibited the PKC activity at the same range of concentrations as it reversed MBG-induced vasoconstriction and Na/K-ATPase inhibition. The cicletanine-induced PKC inhibition was reversed by the same concentration of PDA that reversed the vasoconstrictor action of cicletanine. Thus, the present results demonstrate that the ability of cicletanine to inhibit the PKC is critical for its capacity to antagonize the effects of MBG; in contrast,

Fig. 4



(a) Effect of cicletanine on the activity of rat brain protein Kinase C (PKC) (PepTag assay). Upper panel: horizontal electrophoresis of phosphorylated PKC in a 0.8% agarose gel. Lower panel: concentration–response curve of the inhibitory effect of cicletanine on the PKC activity (ratio of phosphorylated to non-phosphorylated form) from rat brain. (b) Effect of cicletanine on the activity of rat brain PKC (PepTag assay) in the presence of PDA. Upper panel: horizontal electrophoresis of phosphorylated PKC in a 0.8% agarose gel. Lower panel: concentration–response curve of the inhibitory effect of cicletanine on the PKC activity (ratio of phosphorylated to nonphosphorylated form) from rat brain – 100 mmol/l cicletanine inhibits PKC from rat brain (black bar), in the presence of 50 nmol/l PDA, cicletanine-induced PKC inhibition does not occur. Each point represents the mean ± S.E.M from four measurements.

cicletanine-induced relaxation of ET-1-induced contractions is more likely due to the inhibition of low-K_m cGMP-PDE. Previous investigations of the vasorelaxant action of cicletanine found that at high concentrations cicletanine inhibited PKC from monkey aorta (IC_{50} = 900 μmol/l) [6]. In the present study, however, cicletanine exhibited a much more potent PKC inhibitory activity (IC_{50} = 45 ± 11 μmol/l). Since PKC isoforms vary with respect to their sensitivity to different PKC inhibitors [27], we hypothesize that the difference between the prior and present study may reflect a different PKC isoform expression in monkey aorta and rat brain. The specific PKC isoform target(s) for cicletanine remains to be established.

A second new finding of the present study is that while cicletanine alone produced very modest inhibition of the Na/K-ATPase from mesenteric artery sarcolemma, it substantially attenuated the Na/K-ATPase inhibitory action of MBG. This effect of cicletanine was sensitive to a PKC activator, PDA. Various vasoactive substances, both vasorelaxants and vasoconstrictors, can modify Na/K-ATPase activity via its phosphorylation/dephosphorylation by protein kinases [28–30]. Notably, protein kinases phosphorylate the sodium pump in an isoform-specific fashion. The PKC-specific phosphorylation Na/K-ATPase domain is associated with the α-1 isoform [31]. We have previously demonstrated that, in rat aorta, MBG exhibits greater affinity to the α-1 than to the α-3 isoform [13]. Although the state of phosphorylation of the Na/K-ATPase can affect cardiac glycoside binding to this enzyme [32], it is still unknown whether phosphorylation of the Na/K-ATPase by PKC affects the inhibitory activity of endogenous digitalis-like inhibitors, i.e. MBG. However, the present results, showing that pre-treatment of the mesenteric artery rings with H7 abolishes contractile responses to MBG, and that MBG antagonism by cicletanine is PKC-dependent, are consistent with this notion.

In conclusion, the present results demonstrate that cicletanine, via inhibition of PKC, reverses vasoconstriction and Na/K-ATPase inhibition induced by a putative endogenous Na-pump ligand, MBG. Further, our findings indicate the importance of PKC in concurrent modulation of vascular tone via Na/K-ATPase–cardiotonic steroid interactions. Additionally, PKC and Na/K-ATPase are both involved in a common hypertrophic signaling pathway during chronic hypertension [33]. The endogenous and exogenous digitalis-like cardiotonic steroids exert growth-promoting effects [34,35]. Since cicletanine exhibits anti-proliferative ac-

tivity [36] and promotes vascular protection in hypertension [37], the cicletanine–PKC interactions on growth merit further study.

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