Antispasmodic effects of the essential oil of Croton nepetaefolius on guinea-pig ileum: a myogenic activity

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INTRODUCTION

Croton nepetaefolius Baill. (Euphorbiaceae) is an aromatic plant, with a rich essential oil content (1–3% of plant dry weight), that is endemic to north-eastern Brazil, where it is popularly named ‘marmeleiro sabia’ [1]. Infusion and tea made of barks and leaves of C. nepetaefolius are commonly used in folk medicine in the north-east of Brazil as a stomachic, carminative, and intestinal antispasmodic [1,2].

Previous studies from our laboratory have shown that the essential oil of C. nepetaefolius (EOCN) dose-dependently decreased mean aortic pressure (MAP) and heart rate (HR) in both pentobarbital-anesthetized and conscious normotensive rats [3]. Whilst EOCN-induced bradycardia appeared to depend upon the presence of 

ABSTRACT

The effects of essential oil of Croton nepetaefolius (EOCN) on guinea-pig-isolated ileum were studied. We previously demonstrated that EOCN induced reversible relaxation of ileal tone artificially increased by high [K+] nutrient solution. This study aimed to elucidate whether these effects were caused by indirect, neural, or primarily by myogenic effect. EOCN (40 µg/mL) induced a relaxation of basal tone corresponding to approximately 38% of the reference contraction (K+ 60 mM), and was unaltered by 0.5 mM hexamethonium, 0.5 µM tetrodotoxin, 1 µM indomethacin, and 100 µM L-nitroarginine methyl ester (L-NAME). Epinephrine- (100 µM) and EOCN-induced maximal relaxation of ileum pre-contracted with 60 mM KCl represented 16.8 ± 2.3% (n = 10) and 95.0 ± 6.4% (n = 6) of KCl-induced contraction, respectively. EOCN (200 µg/mL) had no effect on the transmembrane resting potential (E_m) of ileum in nutrient solutions with normal (5 mM) and high (80 mM) [K+]. EOCN similarly inhibited the contractions induced by KCl, acetylcholine (ACh) and histamine (IC50 values of approximately 18, 28 and 21 µg/mL, respectively). EOCN also inhibited both the nifedipine-insensitive component of ACh-induced contraction and the contraction induced by ACh in Ca2+-free solution. EOCN accelerated the reversal of a KCl-induced tonic contraction upon withdrawal of Ca2+ from the extracellular medium. Our results suggest that EOCN induces relaxation of guinea-pig ileum by a direct action on smooth muscle via a mechanism largely independent of alterations of E_m and Ca2+ influx, possibly at the level of the contractile apparatus.
an intact and functional parasympathetic nerve drive to the heart, EOCN-induced hypotension appeared independent of the presence of an operational sympathetic nervous system [3]. In deoxycorticosterone-acetate (DOCA)-salt hypertensive and uninephrectomized control, conscious rats, EOCN also decreased MAP and HR in a dose-related manner [4]. Treatment with DOCA-salt significantly enhanced EOCN-induced decreases in MAP without affecting bradycardia. In isolated thoracic aorta preparations from DOCA-salt hypertensive rats, EOCN induced a reduction of phenylephrine-induced contraction. Arteries from DOCA rats showed enhanced sensitivity to EOCN, when compared with uninephrectomized controls. This enhancement appeared to be related mainly to an increase in EOCN-induced vascular smooth muscle relaxation rather than to enhanced sympathetic nervous system activity in this hypertensive model [4]. These data thus supported the hypothesis that EOCN may be a direct vasorelaxant agent acting hypotensively by a mechanism, probably myogenic, that turns to be more active in hypertensive rats.

In isolated guinea-pig trachea. EOCN fully relaxed preparations pre-contracted with 60 mM K⁺ [5]. These data were also compatible with the hypothesis that EOCN acts as an antispasmodic agent by a mechanism that is probably myogenic, because this [K⁺] blocks nerve excitability [6].

EOCN also demonstrated myorelaxant and antispasmodic activities on intestinal smooth muscle in vitro, including on preparations maintained in presence of 60 mM [K⁺]. an action shared by several of its main constituents including 1,8-cineole, methyl-eugenol and terpineol [7,8]. In vivo, EOCN also showed an interesting profile of intestinal myorelaxant and antispasmodic activity in stomach sphincters and small intestine, eliciting a depressive action more potent on basal tone than on spontaneous motility and (at a range of doses) preserving intestinal transit velocity while relaxing the viscera [8].

These results with a profile of action that make EOCN a promising agent with potentiality to therapeutic use, coupled with the suggestion by the previous studies of a myogenic activity, prompted the necessity of the study of its antispasmodic mechanism. As it is known that the patterns of intestinal motility are largely created and controlled by the enteric nervous system, in the present study we investigated whether previously reported EOCN effects on intestinal motility are mediated through a primary action on intestinal intrinsic neurons or through a myogenic effect and tried to elucidate its mechanism of action.

**MATERIALS AND METHODS**

**Plant material**

EOCN was kindly provided by the Department of Organic and Inorganic Chemistry of Federal University of Ceará. EOCN extraction and analysis were as previously described [3,4,5]. Briefly, EOCN was extracted from dry leaves by steam distillation and analysed by gas chromatography (GC) and mass spectrometry (MS) (Hewlett-Packard 6971 GC/MS, Palo Alto, CA, USA) and had the following chemical composition (in % of oil weight): 1,8-cineole (25.4%), methyl-eugenol (14.9%), xanthoxilin (10.1%), β-caryophyllene (9.7%), sabine (5.2%), α-terpineol (5.0%) and other minor constituents. A voucher specimen (no. 3185) of *C. nepetaefolius* is deposited in the Herbarium Prisco Viana of the Federal University of Ceará.

**Solutions and tissue preparation**

Male guinea pigs, weighing 250–400 g, were stunned and killed by cervical dislocation and 2-cm pieces of the ileum were dissected from the ileum segment 10–20 cm near to the ileocecal valve. The material was mounted for tension recording and allowed to equilibrate for 1–2 h in 10-mL chambers containing modified Tyrode solution (composition in mM: NaCl 136.0, KCl 5.0, MgCl₂ 0.98, CaCl₂ 2.0, Na₂HPO₄ 0.36, NaHCO₃ 11.9 and glucose 5.5), pH 7.4, maintained at 37 °C, and bubbled with air.

In solutions with high [K⁺], Na⁺ was simultaneously decreased to maintain isosmolarity. In experiments with calcium-free medium, CaCl₂ was omitted from the normal Tyrode solution and 0.2 mM ethylene glycol-bis(β-aminoethyl ether)N,N,N’,N’-tetraacetic acid (EGTA) was added. Acetylcholine (ACh), and histamine solutions were prepared by adding the substance directly to Tyrode solution. EOCN was initially diluted directly in Tyrode, sonicated, and added to the bath at an amount to reach the final desired concentration. The initial EOCN solution was usually five to 10 times the final concentration, at which dilution level, in all cases, solution transparency (at eye inspection) was identical to Tyrode alone. All reagents and drugs used in this study were of analytical grade, purchased from Sigma Chemical Co. (St Louis, MO, USA), Merck (Darmstadt, Germany) or Reagen (Rio de Janeiro, RJ, Brazil).

**Measurement of contractile activity**

The mechanical responses in ileum were isometrically recorded with a force transducer (Grass, FT03, Quincy, MA, USA) and polygraph (Grass, model 5D). The contractile amplitude was measured at the peak deflec-
tion. For the EOCN-induced relaxation of preparations pre-contracted with 60 mM K+ solutions, only preparations with an at least 5-min lasting plateau contraction were used.

**Measurement of electrical activity**
The transmembrane electrical potential was measured with glass microelectrodes (50–100 MΩ tip resistance) filled with 3 M KCl and connected by a chloridized silver wire to the input of an electrometer (Axoclamp 2B; Axon Instruments, Union City, CA, USA) and monitored on an oscilloscope (Tektronix, model 5111A, Beaverton, OR, USA). At least 10 cells were randomly impaled in each preparation; five in the presence of EOCN and five were used as controls. In cells with the transmembrane potential oscillating rhythmically, the peak negative voltage was recorded.

**Statistical analysis**
All data are expressed as mean ± SEM. Significance (P < 0.05) of the results was assessed by means of paired or unpaired Student’s t-test and ANOVA, followed by a multiple comparison test, where appropriate. EC50 and IC50 values (i.e. the concentration of agonist or antagonist, respectively, at which 50% of the maximal response was observed) were calculated by interpolation from semi-logarithmic plots.

**RESULTS**

**Relaxant effects of EOCN on basal tone of guinea-pig-isolated ileum**
The addition of EOCN (40 µg/mL) elicited a relaxation of the spontaneous tone of the ileum with a maximal effect corresponding to 37.9 ± 4.7% (n = 23) of the amplitude of the contraction induced by 60 mM K+. This effect was fully reversible upon EOCN removal in all cases (Figure 1a).

Previous exposure of preparations to hexamethonium (0.5 mM for 10 min), tetrodotoxin (0.5 µM for 30 min), indomethacin (1 µM for 30 min) and l-nitroarginine methyl ester (l-NAME; 100 µM for 30 min) did not significantly alter the relaxation of basal tone induced by EOCN (40 µg/mL; Figure 1b).

**Inhibitory effects of EOCN on the contractions induced by ACh, histamine, and KCl in the guinea-pig-isolated ileum**

Pre-exposure of the preparation for 10 min to a given EOCN concentration decreased subsequent contractions induced by 60 mM K+, and by sub-maximal (inducing responses approximately 70% of the maximum) concentrations of ACh (0.1–0.5 µM), and histamine (0.2–0.6 µM), with IC50 values of 18.2 ± 2.3 µg/mL (n = 6), 28.0 ± 4.9 µg/mL (n = 8) and 21.6 ± 4.7 µg/mL (n = 6), respectively (Figure 2). There was no significant difference between the IC50 values (P > 0.05; ANOVA) for the inhibitory effect of EOCN on ACh, histamine and KCl-induced contractions.

**Comparison of the relaxant effects of EOCN and epinephrine in the guinea-pig-isolated ileum**

As the activation of intestinal adrenergic receptors may represent a putative pathway for the mediation of the EOCN-induced ileum relaxation, the response of preparations pre-contracted with 60 mM K+ to supra-maximal concentrations of EOCN were compared with those elicited by epinephrine. Whilst epinephrine (10, 20 and 100 µM) relaxed the preparations by amounts that did not significantly differ among themselves (P > 0.05: Bonferroni’s test) and were never superior to
16.8 ± 2.3% \((n = 10; \text{epinephrine } 100 \mu M)\), EOCN \((40 \mu g/mL)\) reduced the tone by 95.0 ± 6.4% \((n = 6)\) of the \(K^+\)-induced contraction, a value significantly greater \((P < 0.001; \text{Bonferroni’s test})\) than the maximal response to epinephrine (Figure 3).

**Effects of EOCN on the transmembrane potential of the isolated ileum**

The transmembrane potential of ileal smooth muscle in normal Tyrode solution \((5 \text{ mM } K^+)\) was not altered by EOCN, with mean values of \(-49.6 \pm 1.7 \text{ mV} (n = 33)\) and \(-50.9 \pm 1.3 \text{ mV} (n = 22)\) \((P > 0.05; \text{unpaired Student’s } t\)-test) in the absence and presence of EOCN \((200 \mu g/mL)\), respectively. In the presence of 80 \text{ mM } K^+, EOCN was also without effect on the transmembrane potential: \(-16.0 \pm 1.1 \text{ mV} (n = 21)\) and \(-17.6 \pm 1.0 \text{ mV} (n = 22)\) \((P > 0.05; \text{unpaired Student’s } t\)-test) in the absence and presence of EOCN \((200 \mu g/mL)\), respectively.

**EOCN-induced inhibition of the nifedipine-insensitive component of ACh-induced contraction of the guinea-pig-isolated ileum**

In the presence of nifedipine \((1 \mu M)\), a concentration that fully blocked the 60 \text{ mM } K^+-induced contraction of the isolated ileum under normal \((Ca^{2+}\)-containing) Tyrode solution (Figure 4a), a supramaximal concentration of ACh \((60 \mu M)\) produced a contraction corresponding to 102.4 ± 10.6% \((n = 6)\) of the \(K^+\)-induced control response. EOCN \((80 \mu g/mL)\) reduced the amplitude of this nifedipine-insensitive component, diminishing its amplitude significantly to 12.1 ± 3.6% of the contraction obtained in the absence of EOCN (Figure 4b; \(P < 0.001; \text{paired Student’s } t\)-test). This effect was reversible upon washout of EOCN.

**Inhibitory effects of EOCN on ACh-induced contraction of the guinea-pig isolated ileum under calcium-free conditions**

The amplitude of the contraction induced by ACh \((60 \mu M)\) in \(Ca^{2+}\)-free Tyrode solution \((\text{with } 0.2 \text{ mM EGTA})\) was progressively reduced with the number of contractions, suggesting \(Ca^{2+}\) release from intracellular stores (Figure 5a). The first contraction (applied at a regular time interval of 5-min after \(Ca^{2+}\) removal) in the absence of \(Ca^{2+}\) was 74.1 ± 5.8% \((n = 6)\) of the control

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**Figure 2** Concentration-dependent inhibitory effects of the essential oil of *Croton nepetaefolius* (EOCN) on submaximal contractions induced by acetylcholine (ACh, 0.1–0.5 \(\mu M\), \(n = 8\)), histamine (HA, 0.2–0.6 \(\mu M\), \(n = 6\)) and \(K^+\) (60 \text{ mM}, \(n = 6\)) (mean ± SEM).

**Figure 3** Comparison of the relaxant effects of the essential oil of *Croton nepetaefolius* (EOCN) and epinephrine in the contraction induced for 60 \text{ mM } K^+ in guinea-pig isolated ileum. (a) Representative trace of the relaxation induced for 10, 20 and 100 \(\mu M\) of epinephrine (Epi 10, Epi 20 and Epi 100, respectively), and 40 \(\mu g/mL\) of EOCN (EOCN 40). (b) Epinephrine and EOCN mean relaxant effects on the reversal of the 60 \text{ mM } K^+-induced contraction (mean ± SEM). ***Significantly different from Epi 10, 20 and 100 \(\mu M\), \(P < 0.001\), Bonferroni’s test \((9 \geq n \geq 11 \text{ for Epi and } n = 6 \text{ for EOCN}).

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response to 60 mM K\(^+\) under normal (Ca\(^{2+}\)-containing Tyrode) conditions. EOCN (80 \(\mu\)g/mL) further reduced the amplitude of this first contraction in Ca\(^{2+}\)-free solution, significantly diminishing its amplitude to 11.4 ± 3.9% of K\(^+\)-induced control contraction (\(P < 0.001\); paired Student’s t-test; Figure 5b). This effect was reversible on washout of EOCN.

**EOCN-induced potentiation of the \(t_{1/2}\) for relaxation due to extracellular calcium removal of ileal preparations exposed to high [K\(^+\)]**

In order to verify whether EOCN could cause a modification of these velocities of relaxation coherent with the hypothesis that EOCN effects may be partially due to a decrease of sensitivity of the contractile machinery to intracellular [Ca\(^{2+}\)], we carried out two protocols similar to those described by Yanagisawa et al. [9] in the presence and absence of EOCN (Figure 6). The control \(t_{1/2}\) values obtained with the first (the relaxation of contraction upon Ca\(^{2+}\) removal in presence of 90 mM [K\(^+\)] and with addition of 0.2 mM EGTA) or with the second protocol ([K\(^+\)] decrease from 90 to 5 mM in presence of 2.0 mM Ca\(^{2+}\)) were 68.5 ± 10.5 s (\(n = 25\)) and 15.3 ± 3.6 s (\(n = 8\)), respectively, which were significantly different (\(P < 0.05\); Dunn’s test). To test the effect of EOCN on the velocity of relaxation, a concentration of 5 \(\mu\)g/mL of this essential oil was chosen because it induced a minimal steady-state reduction of 90 mM KCl-induced tone. In the continuous presence of 5 \(\mu\)g/mL EOCN, the \(t_{1/2}\) of the relaxation upon Ca\(^{2+}\) removal (first protocol) was significantly reduced to 28.0 ± 4.1 s (\(n = 17\); \(P < 0.05\); Dunn’s test), whilst \(t_{1/2}\) for relaxation upon [K\(^+\)] reduction to 5 mM (second protocol) was not significantly altered. The effect of calcium channel blocker on the relaxation observed in

*Figure 4* Inhibitory effect of the essential oil of *Croton nepetaefolius* (EOCN) on nifedipine-insensitive acetylcholine (ACh)-induced contraction of the guinea-pig isolated ileum. (a) Representative trace showing that 60 mM KCl-induced contraction (K60) was blocked in the presence of nifedipine (Nif; 1 \(\mu\)M), whilst there was a tonic component of the ACh (60 \(\mu\)M) response resistant to the presence of the calcium channel blocker. This nifedipine-insensitive component was almost abolished by 80 \(\mu\)g/mL EOCN and fully recovered after wash. (b) Nifedipine-insensitive ACh-induced contraction of the guinea-pig isolated ileum in the absence and presence of EOCN (mean ± SEM) (\(n = 6\)). ***\(P < 0.001\), paired Student’s t-test.

*Figure 5* Inhibitory effect of the essential oil of *Croton nepetaefolius* (EOCN) on the acetylcholine (ACh)-induced contraction of the guinea-pig isolated ileum maintained in Ca\(^{2+}\)-free medium with 0.2 mM EGTA (O[Ca\(^{2+}\)]\(_o\)). (a) Representative trace showing the inhibitory effect of EOCN on the contraction elicited by ACh (60 \(\mu\)M) under O[Ca\(^{2+}\)]\(_o\) conditions. This effect was reversible on washout of EOCN. (b) Inhibitory effect of EOCN on the [Ca\(^{2+}\)]\(_o\)-independent component of the ACh-induced contraction of the guinea-pig isolated ileum (mean ± SEM) (\(n = 6\)). ***\(P < 0.001\), paired Student’s t-test.
the first protocol was also tested. In the continuous presence of nifedipine (0.005 µg/mL), which also minimally relaxed the steady-state K⁺ contraction, the $t_{1/2}$ of relaxation upon Ca²⁺ removal was 53.4 ± 9.9 s ($n = 8$), which was not significantly different ($P > 0.05$; Dunn’s test) from that observed in the first protocol control value (68.5 s).

**DISCUSSION**

We have demonstrated that EOCN reduces basal tone of the isolated guinea-pig ileum, with the maximum amplitude of relaxation similar to that of the well-characterized smooth muscle relaxant papaverine [8]. The present study has confirmed these initial observations and has additionally addressed the hypothesis of EOCN activity on the enteric nervous system as the underlying mechanism of its antispasmodic action. Such investigation turned to be relevant since recent studies in our laboratory have demonstrated that terpineol, an important constituent of EOCN, have depressive effect on nerve activity [10]. The demonstration that the myorelaxant and antispasmodic activity of EOCN is myogenic and independent of neural activity is a novel finding.

The possibility of an indirect action of EOCN on transmitter release from nerve terminals is unlikely, as its relaxant effect was neither altered by tetrodotoxin, a blocker of membrane Na⁺ channels [11,12], nor by the neuronal ganglion blocker hexamethonium [13]. Thus, a participation of the enteric nervous system in the effects of EOCN does not seem likely. This conclusion is supported by the observation that EOCN is capable of completely reversing the tone elicited by high concentrations (60 mM) of KCl. In this situation, the plasma-lamellar membrane of enteric neurons would be sufficiently depolarized ($E_m = -20$ mV) [14] to have sodium channels inactivated and to prevent the generation of action potentials and thus neuronal transmission would be blocked. Instead, the evidence suggests a direct effect of EOCN on the smooth muscle.
It appears improbable that a hyperpolarization of the resting membrane potential in the guinea-pig ileum constitutes its primary mechanism of action. In fact, a concentration of EOCN (200 µg/mL) that produced a maximal relaxation of the ileum caused no significant changes in membrane potential. This contrasts with established smooth muscle relaxants such as cromakalim, a potassium channel activator, which can hyperpolarize smooth muscle close to the K⁺-equilibrium potential (~80 to ~90 mV) [14]. In addition, the fact that EOCN was able to completely relax the contraction induced by high potassium concentration without altering the membrane potential supports this hypothesis and indicates that the profile of action of EOCN is completely different from that of the potassium channel openers, which are unable to relax high potassium concentration-induced contractions in smooth muscle [15,16].

Previous research has shown that activation of adrenoceptors in ileal smooth muscle leads to relaxation [17]. The possibility that such an action may underlie the effects of EOCN appears unlikely, as in our preparations adrenaline was only able to maximally relax the KCl-induced contraction by approximately 16%, whereas EOCN was able to completely revert this response. Similarly, the possible involvement of other mediators of smooth muscle relaxation was excluded. For example nitric oxide (NO) has been shown to tonically inhibit small intestinal motility [18]; however, the relaxant action of EOCN in these conditions was resistant to the effects of the NO synthase inhibitor L-NAME. In addition, it does not appear that the relaxant action of EOCN is due to the release of prostaglandins as its effect was unaffected by the cyclo-oxygenase inhibitor indomethacin.

The possibility that the antispasmodic effects of EOCN were simply caused by a blockade of Ca²⁺ entry via voltage-dependent Ca²⁺ channels was examined by assessing any inhibitory action on the nifedipine-resistant portion of ACh-induced contraction and the ability to block the transient contraction induced by ACh in Ca²⁺-free solution. ACh-induced contraction in smooth muscle is mediated by a release of intracellular Ca²⁺ from the sarcoplasmic reticulum and by Ca²⁺ entry via voltage-sensitive Ca²⁺ channels (VDCCs) by direct or indirect action. EOCN showed no obvious selectivity between agonists in its antispasmodic effects, as contractions induced by ACh and histamine were reversibly blocked with similar IC₅₀ values. In addition, KCl-induced contraction was also inhibited within a similar concentration range, suggesting that EOCN relaxation was not because of plasmalemmal receptor antagonism and may reflect an ability to depress ileal smooth muscle contraction at some stage distal to the receptor transduction process, possibly via direct interaction with contractile proteins or at some level of their intracellular control.

As a means to approach the problem of the mechanism of action of EOCN, we adopted the protocol developed by Yanagisawa and Okada [9], who demonstrated that complete withdrawal of Ca²⁺ from a high (90 mM) KCl concentration solution induced relaxation of the KCl-induced tone in rat aorta more slowly than reduction of K⁺ concentration to 5 mM (maintaining extracellular Ca²⁺ constant at 2.0 mM). Simultaneous measurement of contraction and intracellular Ca²⁺ suggested that this phenomenon was due to a difference in the Ca²⁺ sensitivity of the contractile proteins under the two experimental conditions. The authors concluded that, in KCl-depolarized smooth muscle, the Ca²⁺ sensitivity of the contractile elements may be increased. In the present study, the contractile data generated from these protocols in guinea-pig ileum closely agreed with the previous study in rat aorta, as the t₁/₂ values for relaxation of KCl-induced tone by withdrawal of Ca²⁺ and K⁺ were approximately 68 and 15 s, respectively, compared with 68 and 33 s in the aorta [9]. This suggests that this phenomenon may be a common feature of both vascular and non-vascular smooth muscle. Under this experimental approach when a low concentration of EOCN, which had only slight relaxant effects per se, was added prior to withdrawal of Ca²⁺, the t₁/₂ for relaxation was much greater compared with control values and attained a value similar to the t₁/₂ induced by withdrawal of KCl. This striking ability of EOCN to decrease the t₁/₂ for relaxation upon Ca²⁺ withdrawal was not simply because of an additive relaxant action, as a plateau relaxant response had been obtained with EOCN before withdrawal of Ca²⁺ from the Tyrode solution. Thus, these findings may suggest that EOCN is somehow capable of eliciting a ‘desensitization’ of the contractile apparatus to Ca²⁺.
CONCLUSION
The present study shows that EOCN is able to relax isolated ileum and to inhibit contractions induced by receptor-dependent and -independent mechanisms. The mechanism appears complex, although it seems to be largely independent of membrane potential, influx of extracellular Ca^{2+} and possibly reflects an ability of EOCN to decrease the sensitivity of the contractile proteins to calcium.

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