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Antispasmodic effect of 4′-methylepigallocatechin on guinea pig ileum

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ABSTRACT

The antispasmodic effect of 4′-methylepigallocatechin (MEC), which was isolated from *Maytenus rigida* Mart (Celestraceae), was investigated in vitro in guinea pig intestinal segments. In the isolated ileum, MEC (1 nM–100 μM) did not modify the ileal spontaneous tonus or the electrically elicited contractions. MEC (8 μM) significantly ($p<0.01$) reduced the submaximal contractions induced by histamine (2 μM), carbachol (100 μM) and $BaCl_2$ (0.03 M). An additive relaxing action ($p<0.001$) was observed by co-incubation of verapamil (10 nM) and MEC (8 μM). Although MEC (1 nM–100 μM) did not modify the contractions elicited by 60 mM KCl, it significantly reduced the $CaCl_2$ contractile response without changing the EC_{50} (effective concentration of $CaCl_2$ causing 50% of maximum response). In brief, these results show that MEC has a potent ileal spasmolytic effect and blocks spasms induced by specific and nonspecific stimuli. Importantly, the spasmolytic effects were attained at low concentrations and might be related to the symptomatic relief of abdominal pain that is obtained from the use of the *M. rigida* stem bark.

1. Introduction

Flavonoids are polyphenolic compounds that are ubiquitous in nature being found in human diet, beverages and medicinal plants, among others. They are classified in six major classes based on specific structural differences named flavonols, flavones, flavanones, anthocyanidins, isoflavones and catechins. The flavonoids have aroused considerable interest recently because of their potential beneficial effects on human health [1].

Flavonoids are able to produce different patterns of response on smooth muscles. Several flavonoids, representing different subclasses, have been shown to have concentration-dependent relaxant effects on smooth muscle. For instance, (+) catechin has been shown to have bronchodilator, vasodilator and antispasmodic effects [2,3]. On the other hand, several catechin derivatives, such as (−)-epigallocatechin, (−)-epicatechin, (−)-epigallocatechin gallate, (−)-gallocatechin, have shown contractile responses in rat aortic rings [4].

The flavonoid compound 4-methylepigallocatechin (MEC; Fig. 1) is found in a number of medicinal plants [5–7]. Several pharmacological effects of MEC have been reported, such as DPPH radical-scavenger activity, cytoprotective effects, and anti-obesity properties [8,9]. In addition, we have recently described the bioguided isolation of MEC as the major analgesic compound of *Maytenus rigida* Mart (Celestraceae), which is a medicinal plant that is widely used in Brazil to combat colic, diarrhea and abdominal pain [10].

Abdominal pain is commonly caused by powerful contractions of smooth muscles through the actions of the enteric nervous system. Clinically, pain caused by gastrointestinal spasms is generally treated with drugs that induce smooth muscle relaxation [11]. Although *Maytenus rigida* is commonly used to treat abdominal pain and MEC has been ascribed as the main active compound isolated from *M. rigida*, the effect of

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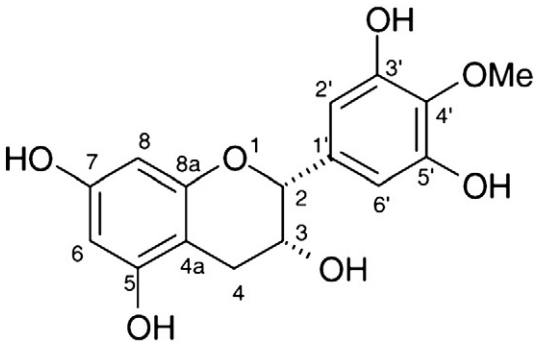

Fig. 1. The chemical structure of 4′-methylepigallocatechin (MEC).

MEC in intestinal smooth muscle has not been investigated before.

Therefore, the present study aimed to investigate the effect of MEC on intestinal smooth muscle. We initially investigated MEC response directly applied in guinea-pig ileum. Moreover, we investigated the effects of MEC on the contractions induced by specific and nonspecific stimuli, such as histamine, carbachol, barium chloride, high potassium concentration and calcium chloride concentration–response curves. MEC response was also evaluated against the contractions elicited by electrical stimuli.

2. Materials and methods

2.1. Tissue preparation

Terminal segments of ileum were mounted as previously described [12]. Isolated segments of ileum were obtained from male and female guinea pigs (300–500 g; $n = 6$–9). Animals were sacrificed by a blow to the base of the skull and cervical dislocation, and 1 cm pieces of the ileum were dissected from the ileum segment 10–15 cm proximal to the ileocecal valve. The material was mounted for tension recording and allowed to equilibrate for 1 h in 10 mL chambers containing aerated normal Tyrode solution (mM: NaCl 137, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2.6H_2O$ 0.5, $NaHCO_3$ 12, NaH_2PO_4 0.4, and glucose 5.5; pH 7.4), and the samples were maintained at 37 °C and bubbled with air. The mechanical response of the ileum was recorded with an isometric force transducer (UC2 model; Gould) linked to a pre-amplifier and computerized data acquisition system (DASA 6600; Gould).

2.2. Protocols

To evaluate MEC response and mechanism of action, the following protocols were carried out.

1. MEC (1 nM–100 μM) cumulative concentration–response curves were obtained in isolated guinea pig ileum in normal Tyrode solution.
2. The submaximal contraction induced by histamine (2 μM) and $BaCl_2$ (0.03 M) was determined in the presence and absence of MEC (8 μM, 20 min, normal Tyrode solution).
3. Submaximal carbachol (100 μM, positive control) responses were determined in the presence and absence of MEC (8 μM), verapamil (10 nM), or verapamil (10 nM) combined with MEC (8 μM). The incubation time was 20 min. The carbachol experiments were performed in normal Tyrode solution.
4. Tissues were incubated with a very low calcium concentration for 30 min. After the incubation, the solution was replaced by a low-calcium, high-potassium depolarizing solution for 10 min. In matched, paired tissues, the cumulative Ca^{2+} concentration–response curves were obtained by increasing the $CaCl_2$ concentration in the organ bath in the presence and absence of MEC (8 μM, 20 min) or verapamil (1 nM, 20 min). The low-calcium solution was prepared by omitting calcium from the Tyrode solution, and the low-calcium depolarizing solution was prepared by omitting calcium from the Tyrode solution and replacing NaCl with an equimolar concentration of KCl.
5. MEC response (1 nM–100 μM cumulatively applied) was determined on the tonic phase of a 60 mM potassium solution. The high-potassium solution was obtained by an equimolar replacement of NaCl by KCl in Tyrode solution. Verapamil (10 nM–10 μM) was used as a positive control.
6. In each preparation, MEC cumulative concentration response curves (1 nM–100 μM) were carried out in the presence of electrically induced contractions (40 V, 0.5 ms, and 0.1 Hz). Morphine (1 μM) was used as a positive control.

2.3. Ethics

The animals were handled humanely in accordance with the guidelines of the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes — ETS No. 123 [13]. The protocols were approved by the Scientific and Bioethics Committee at the Federal University of Sergipe.

2.4. Drugs

MEC was previously isolated from *M. rigida* Mart (Celestraceae) stem bark and physically and spectroscopically characterized [10]. Verapamil, morphine sulfate, carbachol, barium chloride, calcium chloride and histamine were purchased from Sigma Aldrich (USA). Dimethyl sulfoxide (DMSO) was purchased from VETEC (Brazil) and used to dissolve the MEC. For all of the protocols, the response of DMSO was evaluated, and its action could be excluded.

2.5. Data analysis

Contraction amplitudes were measured in at least six independent experiments. Relaxing responses induced by MEC or standard drugs were assessed as a percentage of the maximum height attained in the control curve and expressed as the mean ± SEM. The IC_{50} and EC_{50} were determined by nonlinear regression and expressed as the mean [confidence intervals]. GraphPad Prism 3.0 software was used for the data analysis, and the values were compared using Student's *t* test for paired data or one-way analysis of variance (ANOVA) followed by Bonferroni's test when appropriate. A significance level of 5% was set for all analysis.

3. Results

After 1 h under 1 g of tension, guinea pig ileum suspended in Tyrode solution had stable tension and showed spontaneous contractile activity. In this steady state tonus, MEC (1 nM–100 μM) did not exert contractile or relaxing responses (data not shown).

Fig. 2A shows that the contractions induced by histamine (2 μM) and $BaCl_2$ (0.03 M) were significantly reduced in the presence of MEC (8 μM). The percentages of inhibition for histamine and $BaCl_2$ were 13.6 ± 4.0% ($p<0.01$) and 9.8 ± 2.7% ($p<0.01$), respectively. Both the L-type calcium channel blocker verapamil (10 nM, Fig. 1B) and MEC (8 μM) significantly ($p<0.001$) reduced the contractions induced by carbachol (100 μM). The inhibitory effect of verapamil (10 nM) on carbachol-induced contractions was potentiated ($p<0.05$ vs. verapamil) by co-incubation with MEC (8 μM). The percentages of inhibition were 78.8 ± 2.7% for verapamil, 34.6 ± 9.2% for MEC, and 89.4 ± 2.9% for the combination of verapamil and MEC (Fig. 2B).

$CaCl_2$ in a depolarized medium caused a concentration-dependent contraction in the guinea pig ileum. In paired experiments, $CaCl_2$ concentration–response curves were carried out in the presence or absence of MEC and verapamil. In the first set of paired experiments, the $CaCl_2$ concentration–response control curves showed an EC_{50} of 0.70 [0.57–0.79] mM ($r^2 = 0.98$). As expected, the $CaCl_2$ concentration–response curves were shifted to the right in the presence of verapamil (1 nM, $EC_{50} = 2.0$ [1.7–2.6] mM; $p<0.01$), but the maximum response was not altered (Emax = 104.9 ± 3.9%; $r^2 = 0.97$) (Fig. 3A). Unlike verapamil, MEC (8 μM) did not cause a rightward shift of the $CaCl_2$ concentration–response curves, and MEC did decrease the maximum contractile response to Ca^{2+} (Emax = 88.9 ± 3.7%; $p<0.05$; Fig. 3B). The EC_{50} values

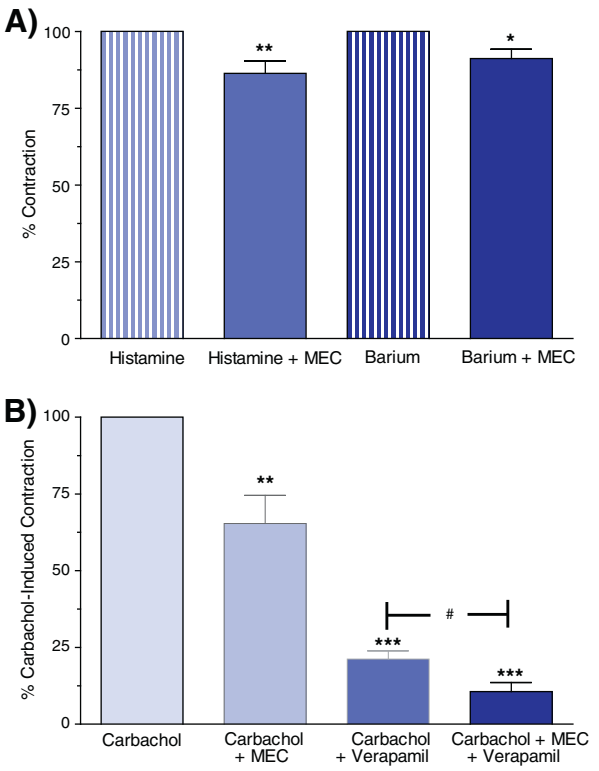

Fig. 2. The spasmolytic effect of MEC during contraction-induced pharmacomechanical and eletromechanical stimuli. A) The spasmolytic effect of MEC (8 μM) during the contractions induced by histamine (2 μM) and $BaCl_2$ (0.03 M). B) The spasmolytic effect of MEC (8 μM), verapamil (10 nM) and the combination of MEC and verapamil (8 μM + 10 nM) during contractions induced by carbachol (100 μM). Results are expressed as the mean ± SEM ($n = 6$–9). $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ vs. histamine, barium chloride or carbachol control contractions, respectively. $^{\#}p<0.001$ carbachol + verapamil vs. carbachol + verapamil + MEC.

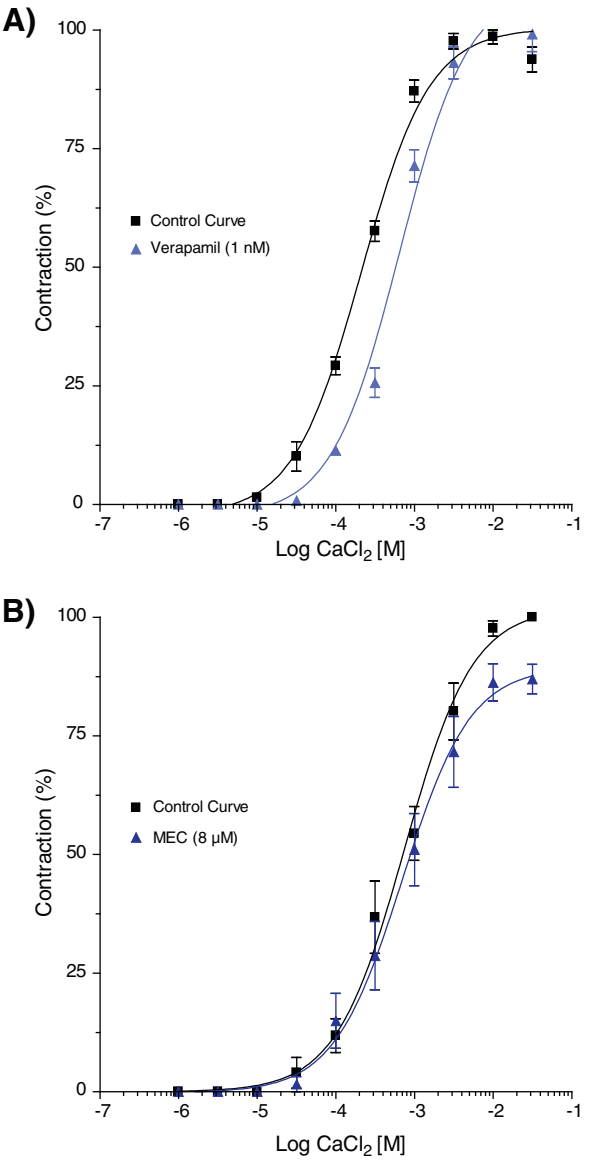

Fig. 3. The spasmolytic effects of MEC during the contractions induced by $CaCl_2$. A) The cumulative concentration–response curves for $CaCl_2$ in the presence and absence of MEC (8 μM). B) The cumulative concentration–response curves for $CaCl_2$ in the presence and absence of verapamil (10 nM). The results are expressed as the mean ± SEM ($n = 6$).

were 1.5 [1.0–2.3] mM ($r^2 = 0.85$) and 1.4 [1.1–1.8] mM ($r^2 = 0.94$) in the absence and presence of MEC, respectively.

KCl (60 mM) produced a sustained tonic contraction that was maintained during the course of the experiments. In the KCl experiments, the calcium channel blocker verapamil (10 nM–10 μM), which was used as a positive control, showed an IC_{50} of 0.10 [0.057–0.19] μM ($r^2 = 0.94$) and an Emax of 100% inhibition (Fig. 4). Interestingly, MEC (1 nM–100 μM) did not reduce the tonic phase of the contractions induced by 60 mM KCl.

In the terminal segments of the guinea pig ileum, electrically induced contractions (0.1 Hz, 0.5 ms, 40 V) were significantly reduced (64.7 ± 4.1% inhibition; $p<0.001$) by morphine (1 μM, positive control). Unlike morphine, MEC (1 nM–100 μM) did not modify the electrically induced contractions in the isolated guinea pig ileum (data not shown).

4. Discussion

The present work was the first study to show that MEC, which was isolated from *Maytenus rigida* stem bark, had a potent spasmolytic effect in the guinea pig ileum and blocked spasms induced by specific and nonspecific stimuli. The spasmolytic effect was attained at low concentrations and might be related to the symptomatic relief of abdominal pain that is observed during the use of *Maytenus rigida* stem bark.

The response of MEC was directly evaluated in the guinea pig smooth muscle. In the absence of contractile agents, MEC did not exert contractile or relaxing responses during the steady state, which indicated that MEC does not modify the intestinal tonus. Interestingly, MEC significantly reduced the contractions elicited by different pharmacomechanical and electromechanical stimuli (i.e., carbachol, histamine and barium) that act on different receptors and signal transduction pathways [14]. These data indicated that MEC acts by a mechanism of action beyond receptor interaction being able to reduce the spasms induced by different neurotransmitters in vivo.

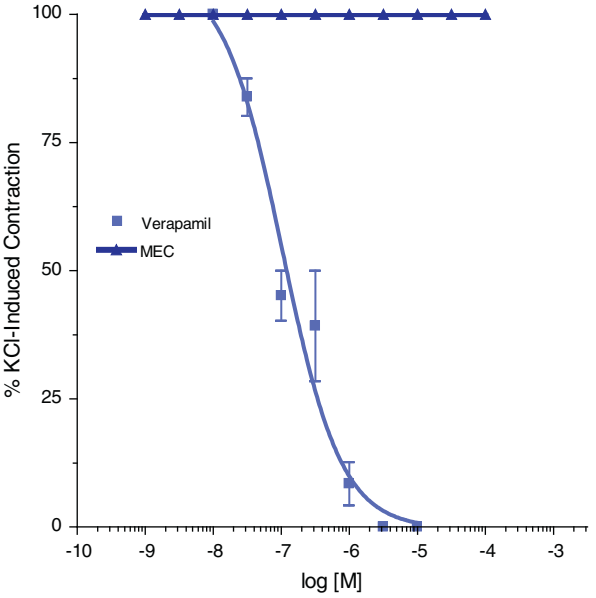

Fig. 4. Investigation of the contractile response to MEC induced by a high potassium concentration (60 mM). A) The cumulative concentration–response curves for MEC (1 nM–100 μM) in the tonic phase of high-potassium-induced contractions. B) The cumulative concentration response curves for verapamil (10 nM–10 μM) in the tonic phase of high-potassium-induced contractions. The results are expressed as the mean ± SEM ($n = 6$).

When isolated strips of gastrointestinal smooth muscle are exposed to muscarinic agonists, the resulting contractions involve an increase in intracellular calcium that is highly dependent on calcium influx and calcium release from intracellular stores. In addition, the contractions that are induced by a high concentration of carbachol (100 μM), which are dependent on intracellular calcium stores, can be reduced, but not abolished, by the L-type calcium channel blocker verapamil [15,16]. In the present work, the contractions induced by a high concentration of carbachol were significantly reduced, but not abolished, by both verapamil and MEC, which suggested, at a first glance, that MEC could be acting as a calcium channel blocker. However, an additive action could be observed after incubating MEC along with verapamil, and a mechanism of action different from the calcium channel blockers could not be immediately discarded.

Several flavonoids have been shown to have concentration-dependent relaxant effects in smooth muscle. Among them, (+) catechin has been shown to have an antispasmodic effect in rodents. Indeed, (+) catechin has been dose-dependently relaxed both spontaneous and high K-induced contraction, and caused a right-ward shift in the Ca^{2+} dose–response curve, in a similar way to observed with verapamil. Therefore, a CCB-like effect has been suggested as the (+) catechin mechanism of action [2]. In the present work, aiming to clarify whether MEC acts as a calcium channel blocker, we compared the effects of verapamil and MEC on $CaCl_2$ concentration–response curves. In this set of experiments, verapamil shifted the $CaCl_2$ concentration–response curves to the right without decreasing the maximum response, whereas MEC reduced the $CaCl_2$ maximum response without shifting the curve to the right. These results supported the idea that MEC and verapamil act through different mechanisms.

In another set of experiments, we have found that verapamil, as expected, reduces the contraction induced by high K^+ concentration whereas MEC did not modify this pattern of contractions. This finding further suggested that MEC and verapamil have different mechanisms of action. On the other hand, this data supports the hypothesis that MEC reduces the sensitivity of the contractile machinery to calcium rather than acting as a calcium channel blocker [15,16]. They also indicated that MEC has a mechanism of action different from that described for (+) catechin.

Epigallocatechin-3-galatte (EGCG), the main polyphenol compound found in green tea, has been shown to have contradictory actions on smooth muscles, being able to both contract and relax aortic rings [17,18]. Because of this contradictory response, EGCG action has been evaluated in rat aortic smooth muscle cells. In this study, it has been shown that EGCG relaxing responses are mediated, at least in part, by an inhibition of phosphodiesterase activity, and subsequent increase in cyclic nucleotide levels, which, in turn, can reduce agonist- or high KCl concentration-induced increases in intracellular calcium [19]. From our results we can suggest that inhibition of phosphodiesterase is a possible mechanism of action of MEC.

However, another study is necessary in order to clarify this matter.

Phloroglucinol is a phenol compound whose structure is embedded in the A ring of MEC. Phloroglucinol has been shown to have an intestinal action similar to that observed for MEC, exerting its main action directly on the intestinal smooth muscle, being a very weak inhibitor of normal peristalsis and relaxing intestinal tract if a spasms exists [20]. Therefore, the phloroglucinol structure present in MEC can contribute to the MEC intestinal effect.

Recently, we reported that MEC has antinociceptive effects in the tail-flick test in rats. Because this effect has been reversed by pretreatment with the opiate antagonist naloxone, we suggested that MEC produces an opiate-like analgesic effect [11]. To investigate the opiate-like effects of MEC, we evaluated the response of MEC on the electrically induced contractions in terminal segments of the guinea pig ileum, where the contractions induced by electrical stimuli are mainly caused by acetylcholine release from enteric neurons. Opiate agonists, such as morphine, are known to reduce the electrically-induced contraction by neuronal opiate receptor activation in terminal segments of guinea pig ileum [21]. MEC, however, did not modify the contractions elicited by electrical stimuli in terminal segments of the isolated ileum, and an opiate-like mechanism of action seems unlikely.

Although polyphenolic compounds in general have been shown to have many potential health benefits when evaluated in vitro, the development of polyphenols for the treatment of several disorders is largely hindered by limited knowledge regarding their stability and bioavailability which can influence the bioactivity in vivo [22]. Although there is still a need for studies on MEC bioavailability, the analgesic effect in rat previously reported indicated that MEC, or even a metabolite, is able to act in vivo [11]. This finding along with the mechanism of action described in the present work fully encourages the development of a medicine based on MEC.

In conclusion, the results of the present work demonstrated that MEC has potent antispasmodic property and blocks spasms induced by specific and nonspecific stimuli. MEC appears to act through a molecular mechanism that reduces the sensitivity of the contractile machinery to calcium. These results provide a pharmacological basis for the efficacy of MEC in hyperexcitability disorders of gastrointestinal smooth muscle.

Acknowledgments

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