INTRODUCTION

Vitamin E is the term used for eight naturally occurring essential fat-soluble nutrients called tocopherols (1-4). Vitamin E is an essential nutrient in the human body that must be provided by foods and its absorption from the intestine is a selective process (3-4). Trolox (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid) is a hydrophilic analogue of vitamin E with a chromane structure similar to \( \alpha \)-tocopherol. The role of vitamin E in human nutrition, health, and disease has broadened and changed over the past two decades. \( \alpha \)-Tocopherol is a phenolic antioxidant, the main lipid soluble antioxidant in the body of lipoproteins and biomembranes (2). Although mainly acting as an antioxidant, vitamin E can also be a pro-oxidant. It can even have non-antioxidant functions: as a signalling molecule, as a regulator of gene expression, and, possibly, in the prevention of cancer and atherosclerosis (4-5).

\( \alpha \)-Tocopherol protects the bladder smooth muscle from the hydrogen peroxide-induced peroxidation (6) and duodenal mucosae from ethanol-induced injury (7). It also attenuates oxidative stress and collagen deposition during the development of experimental chronic pancreatitis (8) as well as nuclear factor kappaB (NF-kB) activation and pro-inflammatory cytokine production induced by lipopolysaccharide (9). Trolox reduces hepatocellular damage (10), protects from ischaemia/reperfusion damage (11), ameliorates the effects of ethanol on acetylcholine-induced response and oxidative stress in isolated rabbit duodenum (12), ameliorates duodenal lipopolysaccharides (LPS)-induced disturbances (13) and abrogates storage-related oxidative stress in small bowel (14).

Some of the cellular actions of \( \alpha \)-tocopherol are independent of its antioxidant ability (15). \( \alpha \)-Tocopherol, but not \( \beta \)-tocopherol, inhibits thrombin-induced protein kinase C activation and endothelin secretion in endothelial cells. \( \alpha \)-Tocopherol has the biological effect of inhibiting the release of proinflammatory cytokines, via inhibition of the 5-lipoxygenase pathway (16-17). The antioxidant effects of vitamin E have been described but the non-antioxidant effects are not well known. In the present work, we propose to study the mechanism of action of Trolox (non-antioxidant effect) on rabbit duodenal motility and contractility.

MATERIALS AND METHODS

Animals

Male New Zealand rabbits, weighing 2–2.5 kg, were fed with standard rabbit food and given free access to water. The rabbits were humanely handled and put down in accordance with the Spanish Policy for Animal Protection RD1201/2005 and the European Union Directive 2010/63/EU.
**Chemicals**

Acetylcholine (ACH), Trolox (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid), Bay K8644 (a L-type Ca<sup>2+</sup> channel activator), apamin (a blocker of small-conductance Ca<sup>2+</sup>-activated K<sup>+</sup> channels, SK<sub>K</sub>), charybdotoxin (a selective blocker of intermediate- and large-conductance Ca<sup>2+</sup>-activated K<sup>+</sup> channels, SK<sub>K</sub>Ca), glibenclamide (a blocker of ATP sensitive K<sup>+</sup> channels), quinine (a blocker of voltage-sensitive K<sup>+</sup> channels), tetraethylammonium chloride (TEA, a non-specific K<sup>+</sup> channels blocker), 2,5-dideoxiodenosine (DOA, an adenosyl cyclase inhibitor) and nimesulide (a cyclooxygenase-2 (COX-2) inhibitor), were obtained from Sigma (Madrid, Spain). 1H-1,2,4]oxadiazolo [4, 3-a]quinoxalin-1-one (ODQ, a guanylyl cyclase inhibitor) was purchased from Tocris (Madrid, Spain). All chemicals were analytical grade. Trolox was dissolved in Krebs solution. Bay K8644 was dissolved in ethanol. Glibenclamide, DOA and ODQ were prepared in dimethylsulfoxide. Apamin was diluted in acetic acid. All other chemicals were dissolved in distilled water.

**Preparation of duodenal segments and experimental protocols**

Segments of rabbit duodenum were removed. Isometric recordings of the longitudinal and circular smooth muscle of the duodenum were performed as described previously (13, 18). Whole thickness segments were vertically suspended in a thermostatically controlled organ bath containing Krebs solution (in mM: NaCl 120, KCl 4.70, CaCl<sub>2</sub> 1.20, NaHCO<sub>3</sub> 24.50, KH<sub>2</sub>PO<sub>4</sub> 1.00 and glucose 5.60) at 37°C to achieve pH 7.4 and continuously gassed with 95% O<sub>2</sub> and 5% CO<sub>2</sub>. Each segment was connected to an isometric force transducer (Pioden UF1, Graham Bell House, Canterbury, UK). The segments were stretched passively to an initial tension of 20 mN. The mechanical activity was amplified (The MacLab Bridge Amp, AD Instruments Inc, Milford MA, USA) with a range of 2 mV and recorded for further analysis using the MacLab Systems software. The segments were allowed to equilibrate in Krebs solution for 45 min before use.

After the adaptation period, the spontaneous contractions of the duodenum and the ACh (0.1 mM) responses were recorded in Krebs solution and considered as the control responses. The inhibitors were added to the bath 15 min before the addition of Trolox for 90 min and then a second ACh (0.1 mM) response was evoked. This last response to ACh was compared with the first response to ACh and expressed as percentage. Each experimental protocol was systematically performed on 4 preparations for 90 min and then a second ACh (0.1 mM) response was evoked. The spontaneous contractions of longitudinal and circular smooth muscle of rabbit duodenum incubated for 90 min in Krebs solution served as its own control.

**Analysis of data**

The amplitude (in mN) and the frequency (contractions per minute, cpn) of spontaneous contractions, and the integrated mechanical activity per second (mN s<sup>-1</sup>), were calculated as previously described (18). Data are presented as mean ± S.E.M. Data sets were compared using one-way variance analysis (ANOVA) tests and P-values were determined using the Scheffe F test. Differences with P-values <0.05 were considered statistically significant.

**RESULTS**

**Spontaneous contractions**

The spontaneous contractions of longitudinal and circular smooth muscle of rabbit duodenum were rhythmic and phasic with an amplitude of 20.1 ± 2.4 mN, and a frequency of 14.2 ± 0.5 cpn and 13.2 ± 0.6 cpn (n=24) respectively. At a concentration of 12 mM, Trolox reduced the amplitude to 1.2 ± 0.4 mN and 1.0 ± 0.2 mN and the frequency to 0.9 ± 0.4 cpn and 3.0 ± 0.7 cpn (n=24) of longitudinal and circular smooth muscle of spontaneous contractions respectively (Fig. 1).

We studied the effect *per se* of the inhibitors used in this study in longitudinal muscle, Bay K8644 (0.01 µM), nimesulide (1 µM), ODQ (1 µM) and DOA (10 µM) reduced the amplitude of spontaneous contractions and ACh-contractions, while nimesulide reduced only the frequency of contractions. In circular muscle, ODQ reduced the amplitude and nimesulide reduced the frequency of spontaneous contractions; DOA reduced the ACh-contractions in duodenum (Table 1). The effects *per se* of the K<sup>+</sup>-channel inhibitors used in this study have been described previously in rabbit duodenum (19, 20).

**Effect of Trolox**

Trolox 12 mM induced a reduction on the amplitude and frequency of spontaneous contractions and on the ACh-contractions, that were reverted by quinine (10 µM) in longitudinal and circular muscles (Figs. 1 and 2).

The Trolox effect on the amplitude of spontaneous contractions was reverted by charibdotoxin (0.01 µM) and glibenclamide (0.1 µM).

**Table 1.** Amplitude and frequency of spontaneous contractions and ACh-induced contractions in longitudinal and circular smooth muscle of rabbit duodenum incubated for 90 min in Krebs solution or in the presence of Bay K8644 (0.01 µM), nimesulide (1 µM), ODQ (1 µM) or DOA (10 µM). The values are the mean ± S.E.M. Data are expressed as a percentage of the amplitude and frequency of spontaneous contractions and integrated mechanical activity to ACh respect to control conditions (Krebs). The number of segments from 4 rabbits is in parentheses.

<table>
<thead>
<tr>
<th></th>
<th>Amplitude of contractions</th>
<th>Frequency of contractions</th>
<th>ACh contractions</th>
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<tr>
<td></td>
<td>Longitudinal muscle</td>
<td>Circular muscle</td>
<td>Longitudinal muscle</td>
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<tr>
<td>Krebs</td>
<td>95.3±4.9 (15)</td>
<td>95.2±6.6 (16)</td>
<td>93.1±3.8 (14)</td>
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<tr>
<td>Bay K8644</td>
<td>59.0±9.5 (9)**</td>
<td>78.0±10.2 (9)</td>
<td>96.6±1.8 (9)</td>
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<tr>
<td>Nimesulide</td>
<td>32.0±7.21 (8)***</td>
<td>66.7±18.2 (7)</td>
<td>38.1±14.3 (8)***</td>
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<tr>
<td>ODQ</td>
<td>44.3±5.8 (8)**</td>
<td>57.4±13.2 (8)*</td>
<td>95.9±10.2 (8)</td>
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<tr>
<td>DOA</td>
<td>68.5±10.5 (8)*</td>
<td>80.6±13.1 (7)</td>
<td>86.6±9.1 (8)</td>
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* P<0.05, ** P<0.01, *** P<0.001
µM) only in circular muscle. Furthermore, Bay K8644 (0.01 µM), apamin (0.1 µM), tetroxalmonium chloride (TEA, 5 mM) and 2,5-dideoxiadenosine (DOA, 10 µM) decreased the Trolox effect on amplitude of contractions in circular muscle, and Bay K8644, charibdotoxin and TEA in longitudinal muscle (Fig. 1).

The effect of Trolox on the frequency of spontaneous contractions was reverted by TEA (5 mM) only in longitudinal muscle. Moreover, Bay K8644 (0.01 µM), charibdotoxin (0.01 µM), glibenclamide (0.1 µM), TEA (5 mM) and 2,5-dideoxiadenosine (DOA, 10 µM) decreased the Trolox effect in circular muscle and Bay K8644 in longitudinal muscle (Fig. 1).

The effect of Trolox on ACh-induced contractions was reverted by Bay K8644 (0.01 µM), ODQ (1 µM) or nimesulide (1 µM) only in circular muscle (Fig. 2). Furthermore, apamin (0.1 µM), charibdotoxin (0.01 µM), glibenclamide (0.1 µM), TEA (5 mM), and 2,5-dideoxiadenosine (DOA, 10 µM) reduced the ACh-induced contractions in circular muscle, and Bay K8644, apamin, charibdotoxin, glibenclamide, DOA, ODQ or nimesulide in longitudinal muscle (Fig. 2).

DISCUSSION

The antioxidant properties of vitamin E or α-tocopherol have been extensively studied in various processes (6-14). In addition, vitamin E has other non-antioxidant actions (21).

![Fig. 1. Effect of the incubation for 90 min with Krebs (K, control) or Trolox (T, 12 mM) on amplitude and frequency of spontaneous contractions of the longitudinal and circular smooth muscle from rabbit duodenum. The effect of Bay K8644 (B, 0.01 µM), apamin (A, 0.1 µM), charibdotoxin (C, 0.01 µM), glibenclamide (G, 0.1 µM), quinine (Q, 10 µM), tetroxalmonium chloride (TEA, 5 mM), 2,5-dideoxiadenosine (DOA, 10 µM), ODQ (0.1 µM), nimesulide (N, 1 µM) added 15 min before Trolox (12 mM) on amplitude and frequency spontaneous contractions. Columns are mean percentage values with respect to spontaneous contractions in Krebs (control), and vertical bars indicate SEM. **P<0.01, *** P<0.001 vs. Krebs. #P<0.05, ## P<0.01, ### P<0.001 vs. Trolox.](image-url)
Vitamin E influences the activity of several enzymes (e.g., protein kinase C (PKC), protein phosphatase 2A (PP2A), COX-2, 5-lipoxygenase, nitric oxide synthase, nicotinamide adenine dinucleotide phosphate-oxidase, superoxide dismutase, phospholipase A2) and modulates the expression of genes that are involved in atherosclerosis (22).

Previously, we have shown that Trolox antagonizes the reduction evoked by ethanol or LPS on the duodenal contractility of rabbit (12, 13). These studies suggest that Trolox acts as an antioxidant agent.

The pattern of intestinal motility is attributed to rhythmic oscillations in the membrane potential of slow waves, and the activity of slow waves is generated in the interstitial cells of Cajal, the pacemaker cells of the intestine (23). The Ca\(^{2+}\) ions participate in the amplitude and frequency of spontaneous contractions of smooth muscle of small intestine (19, 24, 25). The effects *per se* of quinine (a blocker of voltage-sensitive K\(^+\) channels), apamin (a blocker small-conductance Ca\(^{2+}\)-activated K\(^+\) channels), charibdotoxin (a blocker of intermediate- and large-conductance Ca\(^{2+}\)-activated K\(^+\) channels), glibenclamide (a blocker of ATP sensitive K\(^+\) channels) and quinine (a blocker of voltage-sensitive K\(^+\) channels) have been described previously (19, 20). Bay K8644 (a L-type Ca\(^{2+}\) channel activator), nimesulide (a COX-2 inhibitor), ODQ (a guanylyl cyclase inhibitor) and DOA (a adenylyl cyclase inhibitor) reduced the amplitude of
spontaneous contractions of longitudinal muscle and nimesulide decreased the frequency in longitudinal and circular smooth muscle (Table 1).

We studied the possible participation of Ca²⁺- and K⁺-channels and enzymes as adenyl cyclase, guanylyl cyclase and COX-2 on the effect of Trolox in spontaneous contractions and on ACh-induced contractions of longitudinal and circular muscle from duodenum. Our results show that Trolox-induced reduction on the amplitude and frequency of spontaneous contractions was reverted by quinine in longitudinal and circular muscle. Furthermore, chariodotoxin, glibenclamide, apamin, TEA, Bay K8644 or DOA reverted or reduced the amplitude or frequency of spontaneous contractions in longitudinal or circular muscle of duodenum. These results suggest that Trolox reduces the spontaneous contractions by the activation of K⁺ channels (voltage-sensitive, small-, intermediate- and large-conductance Ca²⁺-activated, and ATP sensitive), L-type Ca²⁺ channels and the enzyme adenyl cyclase.

In normoxia, the addition of D α-tocopherol produces no discernible effect on the frequency or magnitude of spontaneous mechanical activity of colonic muscles. However, in hypoxic tissue it elicits contractile activity and restores the levels of frequency and force of spontaneous contractions in normoxia. Moreover, Trolox and vitamin K₃ do not have agonist activity in the hypoxic colon (26). The inhibitors of K⁺-channels cause different effects on tissues. Chariodotoxin reduces Ca²⁺-dependent K⁺ currents in human esophagus (27). The large-conductance Ca²⁺-activated K⁺ channels are constitutively activated for modulations of spontaneous activity of guinea pig ileum longitudinal muscle, but not ATP-regulated K⁺ channels (28). The spontaneous contractions of rat ileum decrease after quinine administration (29). The pretreatment of rabbit duodenum with chariodotoxin, apamin, glibenclamide or ODQ do not affect the terpinen-4-ol induces relaxation (30). Our results of Trolox, in part, are in accordance with studies in which the acute administration of vitamin E reduces infarct size and maintains the beneficial effect of ischemic preconditioning via mitochondrial Kₒᵣ channels and cGMP (31). In myocytes of the guinea pig antrum, S(β)-Bay K8644 enhances the peak amplitude of Ba²⁺ currents, but on the contrary, R(β)-Bay K8644 inhibits Ba²⁺ currents (32). These results agree, in part, with our results of Bay K8644 because it reduced the effect of Trolox on the amplitude and frequency of spontaneous contractions in rabbit duodenum.

In the present study, the diminution of ACh-induced contractions evoked by Trolox was reverted by quinine in the longitudinal and circular muscle and by Bay K8644, ODQ, or nimesulide in circular muscle, being quinine the most potent blocker. Moreover, the effect of Trolox on ACh-induced contractions was decreased in the presence of apamin, chariodotoxin, glibenclamide, TEA or DOA in longitudinal or circular muscle of duodenum. Our results suggest that the in the Trolox effect on ACh-induced contractions participates K⁺ and Ca²⁺ channels, as well as the enzymes guanylyl cyclase, adenyl cyclase and COX-2. In fact, these Ca²⁺ or K⁺ channels and enzymes are involved in intestinal contractions. Quinine does not modify the ACh-contractions in smooth muscle of rabbit duodenum (20). Ca²⁺-free solutions diminish the ACh-contractions in longitudinal and circular muscle of small intestine (25). Activation of Ca²⁺-activated K⁺ channels is involved in duodenal dysmotility induced by ethanol (18).

2. 2-azobis (2-amino2propane) dihydroychloride reduces the amplitude of the spontaneous contractions of rabbit duodenal muscle by inward rectifier and intermediate and large-conductance Ca²⁺-activated K⁺ channels (33). Diocolfenac, a nonsteroidal anti-inflammatory drug, diminishes the delayed-rectifier K⁺ current in NSC-34 neuronal cells and dorsal root ganglion neurons by the activation of M-type K⁺ current (34). Dopamine responses were reduced by adenyl cyclase inhibition on mouse ileum contractility (35). Nimesulide decreases isoprenaline-induced inhibition of postoperative ileus in rat circular jejunal muscle (36). These results agree with our results with nimesulide in duodenum.

In conclusion, our results suggest that the Trolox-induced reduction on the contractility of rabbit duodenum are mediated by K⁺ channels, L-type Ca²⁺ channels, adenyl cyclase, guanylyl cyclase and COX-2.

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