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Terpinen-4-ol: mechanisms of relaxation on rabbit duodenum

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Abstract

The effect of terpinen-4-ol was studied on rabbit duodenum in-vitro. Terpinen-4-ol induced relaxation of the basal tonus (IC50 170.2 (95% confidence interval, 175–204) μM) with a maximal relaxant response of 180.4 ± 3.9% (n = 6) of the contraction induced by 60 mM [K^+]. The maximal relaxation induced in control conditions was not affected ($P > 0.05$) by pretreatment of the tissues with phentolamine (50 μM) or propranolol (10 μM), N^G nitro-L-arginine methyl ester (L-NAME; 1 mM), 1*H*-(1,2,4)-oxadiazolo[4,3-a]quinoxalin-1-one (ODQ; 100 μM), hexamethonium (1 mM), tetrodotoxin (1 μM), the mixture charybdotoxin–apamin (1 μM), glibenclamide (10 μM), 4-aminopyridine (10 μM) or tetraethylammonium (100 μM). In addition, terpinen-4-ol completely relaxed tissues precontracted with 60 mM [K^+] solutions (IC50 325.9 (245.1–433.1) μM) and also blocked (IC50 154.7 (117.7–191.7) μM) the phasic component of this contraction. At a concentration of 195 and 650 μM it reduced by 41.3 ± 3.4% and 75.4 ± 3.1%, respectively the maximal contractile response to Ca^{2+} in depolarized duodenum. Terpinen-4-ol completely blocked the component of carbachol-induced contraction, which was resistent to nifedipine (100 μM) pretreatment or to a Ca^{2+}-free solution. These data show that terpinen-4-ol relaxes intestinal smooth muscle and suggest that this effect is myogenic in nature and depends on calcium antagonism.

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Introduction

Terpinen-4-ol is a volatile, low toxicity monoterpenoid alcohol found in the essential oil of many reputed medicinal plants, in some of which it is an important chemical constituent, such as *Alpinia speciosa* K. Shum, *Melaleuca alternifolia, Camellia sinensis*, *Myrtus communis, Laurus nobilis, Croton sonderianus, Eucalyptus globulus* (Craveiro et al 1981; Oliveira 1994; Carson & Riley 1995; Tisserand & Balacs 1995). Terpinen-4-ol is also a constituent of some pharmaceutical inhalants and syrups that are marketed as over-the-counter products and is also used in aromatherapy (Franchomme & Pénoel 1995). Terpinen-4-ol has been reported to have several pharmacological properties, including anti-inflammatory (Hart et al 2000), antimicrobial (Barel et al 1991; Carson & Riley 1995; Raman et al 1995), antiprotozoal (Mikus et al 2000), acaricidal (Walton et al 2004), potential anti-cancer (Calcabrini et al 2004), antispasmodic and intestinal relaxant (Magalhães et al 1998; Bezerra et al 2000), local anaesthetic (Moreira et al 2001; Ghelardini et al 2001) and antihypertensive (Lahlou et al 2002, 2003) activity. The mechanism of action in the reported intestinal myorelaxant effect of terpinen-4-ol has been investigated. Previous studies on this issue (Magalhães et al 1998; Bezerra et al 2000) were undertaken in our laboratory and related the myorelaxant activity of two crude essential oils (extracted from the leaves of *Croton nepetaefolius* and *Alpinia speciosa* Schum., respectively) to the myorelaxant activity induced by the major components of the essential oil, including terpinen-4-ol. This study aims to evaluate the intestinal myorelaxant and antispasmodic activity of terpinen-4-ol in a preparation with high basal tonus and spontaneous mechanical activity, to better understand its mechanism of action.

Materials and Methods

Materials

Terpinen-4-ol was purchased from Aldrich Chemical Co. Inc. (Milwauke, WI), suspended in Tween 80 (3%), stored refrigerated and diluted daily as necessary. All other drugs and ethylene glycol-bis-(β-amino-ethyl ether) *N*,*N*,*N*′,*N*′-tetra-acetic acid (EGTA) were purchased from Sigma Company (St Louis, MO) and diluted in saline at the time of use. Tetrodotoxin was diluted in citrate buffer at pH 4.0 and 1-mL volumes (1 mM) were stocked refrigerated (−20°C) until used. Other chemicals used were of analytical grade obtained from Reagen and Grupo Química (Rio de Janeiro, RJ, Brazil).

Animals

Experiments were performed on male California white rabbits housed under controlled lighting (12-h cycle) and having free access to food and water. The guidelines for care and use of animals approved by the local institution were followed and conformed to the standards for use of laboratory animals reported in the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication no. 85-23, revised 1996).

Experimental procedure

The rabbits were sacrificed by cervical displacement followed by exsanguination. After rapid excision of the proximal duodenum (15–20 cm long), 4-cm segments were suspended, with a resting tension of 1 g, in 10-mL organ baths containing Tyrode solution (composition in mM: NaCl 137, KCl 5, $CaCl_2$ 1.8, $NaHPO_4$ 0.4, $NaHCO_3$ 15 and glucose 5.5) with the pH adjusted to 7.4, kept at 37°C and gassed continuously with air bubbling. Alteration in resting tension was recorded by means of an isotonic lever (6-fold magnification) on a smoked drum. After an equilibration period of 1 h, tissues were exposed for 1 min to a high-potassium solution (60 mM [K^+]) and the tonic component of this contraction (K^+-contracture) was used as an internal standard control. This high-potassium solution was achieved by equimolar replacement of NaCl by KCl. After washout, a concentration–response curve for terpinen-4-ol was performed under normal conditions (basal tonus) and in K^+ (60 mM)-precontracted tissues and the effect was expressed as percentage of the standard K^+-contracture. The effect of terpinen-4-ol was studied using two paired segments mounted on different baths and cumulatively increasing the concentration of terpinen-4-ol in one bath while adding vehicle, isovolumetrically, to the other. The difference between the baseline of the control and test segment was used to express the relaxation induced by terpinen-4-ol. The relaxation (negative deflection) was measured in mm and expressed as a percentage of the maximal tonic contraction (positive deflection) induced by K^+ (60 mM).

In another set of experiments, the curves obtained under normal conditions (basal tonus) were repeated after a 5-min incubation with phentolamine (50 μM) alone or mixed with propranolol (10 μM), L-nitroarginine methyl esther (L-NAME; 1 mM), 1*H*-(1,2,4)-oxadiazolo[4,3-a]quinoxalin-1-one (ODQ; 100 μM), hexamethonium (1 mM), tetrodotoxin (1 μM), the mixture charybdotoxin–apamin (1 μM), glibenclamide (10 μM), 4-aminopyridine (10 μM) or tetraethylammonium (100 μM). The results were expressed as the percentage of the terpinen-4-ol curves in the absence of the pharmacological blocker.

In another separate series of experiments terpinen-4-ol (6.5–650 μM) was added to the preparation 5 min before the induction of phasic contractions with K^+ 60 mM. The phasic component was considered at the peak deflection after 3–5 s exposure to the high-potassium solution. The response obtained in the presence of each concentration of terpinen-4-ol was expressed as the percentage of the K^+-induced phasic contraction in the absence of terpinen-4-ol.

In other experiments the following protocol was observed: after a 60-min equilibration period the preparation was incubated in a Ca^{2+}-free solution, achieved by omitting $CaCl_2$ in the reference medium, for 30 min and thereafter in a Ca^{2+}-free high-K^+ (60 mM) depolarizing Tyrode solution for 10 min. Cumulative concentration–response curves were then obtained by increasing the $CaCl_2$ concentration in the bath (10^{-7} to 10^{-2} M). $CaCl_2$ was then washed out and the duodenum was re-incubated in a Ca^{2+}-free Tyrode solution for 30 min. The high-K^+ depolarizing procedure was repeated, but terpinen-4-ol (195 and 650 μM) was added to the bath 15 min before the first addition of $CaCl_2$.

We also studied the effect of terpinen-4-ol (195 μM) on the component of carbachol (10^{-4} M)-induced contraction that was resistant to 100 μM nifedipine or that still persisted in a Ca^{2+}-free medium. This contraction was expressed as a percentage of the maximal response obtained in a normal Tyrode solution and the maximal amplitude compared in the absence and presence of terpinen-4-ol.

Statistical analysis

The data were expressed as means ± s.e.m of at least six independent experiments. The difference between groups was verified by using analysis of variance with Tukey as a post-hoc test with 5% significance. The data were analysed using SIGMA STAT software (Sigma Chemical Co., St Louis, MO). The maximal response to terpinen-4-ol in each specific situation was labelled as R_{max}. The EC50 values, defined here as the concentration that induced 50% of the maximal response to terpinen-4-ol, were calculated by using GraphPad Prism 3.00 and were expressed as mean ± 95% confidence interval.

Results

At concentrations in the range 1–400 $\mu g\,mL^{-1}$ (6.5 μM to 2.6 mM) terpinen-4-ol induced a dose-related duodenal relaxation with a maximal amplitude of 180.4 ± 3.9% of K^+-contracture amplitude and an EC50 of 170.2

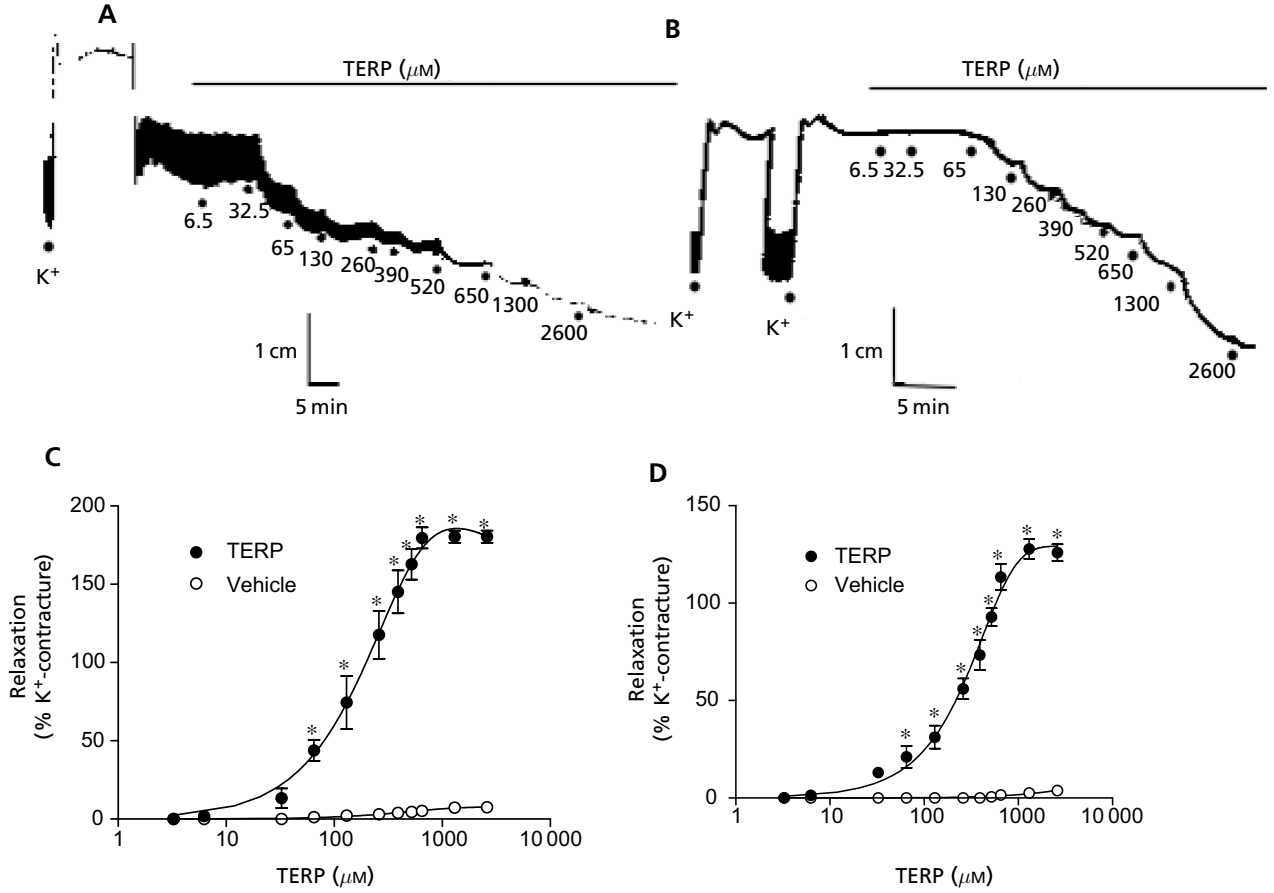

Figure 1 Myorelaxant effect of terpinen-4-ol (TERP) on rabbit duodenum. A, B. Pictures of representative experiments. Preparations at basal tonus (A) and precontracted by exposure to 60 mM [K^+] (B). Below the traces is shown the TERP application (dots) with the corresponding concentration (μM) (numbers below the dots). C, D. Graphs showing quantification (mean ± s.e.m., n = 6) of TERP relaxant effects on duodenum at basal tonus (C) or precontracted by 60 mM [K^+] (D). Vehicle was added isovolumetrically to control parallel baths. Ordinate, relaxation amplitude expressed as percentage of the previous control tonic component of the contraction induced by 60 mM [K^+]. *$P < 0.05$, TERP vs vehicle; analysis of variance followed by Tukey.

(175–204) μM (Figure 1C). The spontaneous mechanical activity of the duodenum was decreased by terpinen-4-ol in a concentration-dependent manner and completely abolished at the maximal concentration. A representative experimental record of the effect of terpinen-4-ol on the duodenum basal tonus is shown in Figure 1A. After terpinen-4-ol washout, duodenal tonus promptly recovered to values not significantly different from control. Moreover, when added cumulatively on tissues tonically contracted with 60 mM [K^+], terpinen-4-ol produced a dose-dependent relaxation with maximal amplitude attained at 125.9 ± 4.3% of the control K^+-contracture and an EC50 of 325.9 (245.1–433.1) μM (Figure 1D). A typical recording of this effect is shown in Figure 1B. This relaxation was also promptly reversed by removing terpinen-4-ol from the bath medium.

The maximal level of terpinen-4-ol-induced duodenal relaxation was not significantly altered (n = 6; $P > 0.05$) by any of the antagonists used (Figure 2A–D), such as phentolamine, propranolol, L-NAME, ODQ, tetrodotoxin, hexamethonium 1 mM, the mixture charybdotoxin–apamin, glibenclamide, 4-aminopyridine or tetraethylammonium. The curves were not shifted and the slope and EC50 values for terpinen-4-ol concentration–response curves also remained unchanged in the presence of the pharmacological blockers used (see Table 1 for R_{max} and EC50 values).

On the other hand, in preparations incubated for 5 min with 6.5, 19.5, 65, 195 or 650 μM terpinen-4-ol, the phasic contractions induced by 60 mM [K^+] were blocked by 1.1 ± 0.6%, 10.7 ± 2.3%, 23.3 ± 1.5%*, 64.2 ± 2.7%* and 95.6 ± 0.3%*, respectively (n = 6; *$P < 0.05$, compared with vehicle-treated tissues) (Figure 3A). The calculated EC50 for this effect was 154.7 (117.7–191.7) μM. In addition, terpinen-4-ol also blocked the Ca^{2+}-induced contraction on depolarized tissue in a concentration-related manner and this effect was not reversed by increasing the Ca^{2+} concentration in the medium. Terpinen-4-ol, 195 and 650 μM, decreased the maximal response to calcium by 41.3 ± 3.4% and 75.4 ± 3.1%, respectively (Figure 3B).

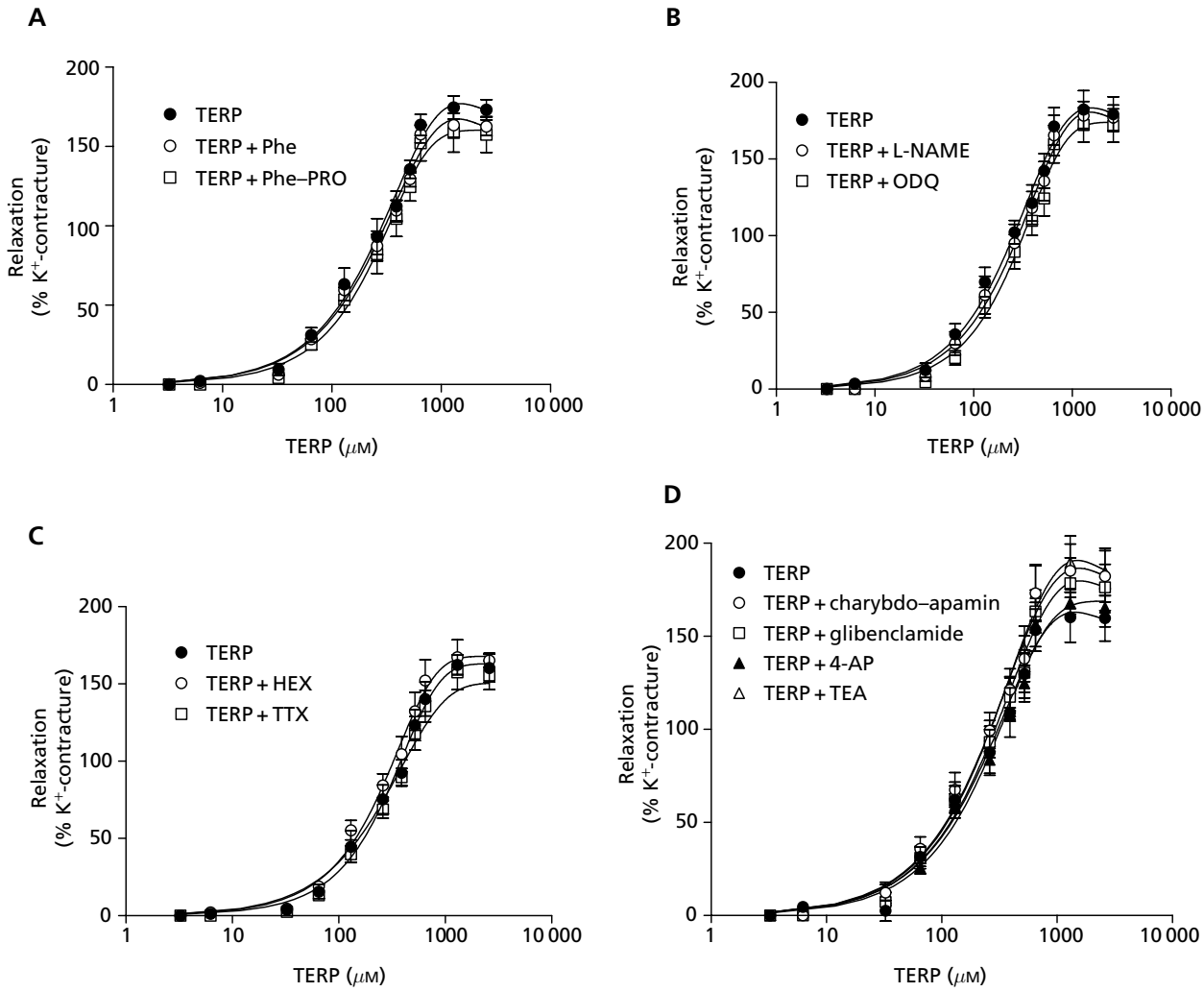

Figure 2 A. The effect of an α-adrenergic blocker phentolamine (10 μM) or the combination of phentolamine and a β-adrenergic blocker (propranolol 10 μM) in the relaxant response of the rabbit duodenum to terpinen-4-ol (TERP). B. The impact of a nitric oxide synthase inhibitor of soluble guanylyl cyclase (ODQ). C. The effect of blocking neuronal sodium channels or autonomic ganglia in the myoenteric plexus with hexamethonium. D. The effect of blocking different subtypes of potassium channel. The data are expressed as mean ± s.e.m. of six isolated experiments (n = no. of rabbits used). Phe, phentolamine; PRO, propranolol; L-NAME, N^G nitro-L-arginine methyl ester: ODQ, 1*H*-(1,2,4)-oxadiazolo[4,3-a]quinoxalin-1-one; HEX, hexamethonium; TTX, tetrodotoxin: TEA, tetraethylammonium.

Terpinen-4-ol (195 μM) reversibly blocked, in the order of 95.2 ± 2.1% (n = 7; $P < 0.05$), the component of carbachol (100 μM)-induced contraction, which was resistant to nifedipine at a concentration (100 μM), that fully blocked the 60 mM [K^+] contracture (Figure 4B, C). Similarly, terpinen-4-ol (195 μM) reversibly blocked (92.0 ± 4.9%; n = 5; $P < 0.05$) the contraction elicited by carbachol in a Ca^{2+}-free nutrient solution enriched with EGTA (Figure 4A, D).

Discussion

The intestinal relaxation induced by terpinen-4-ol is consistent with previous studies undertaken in the guinea-pig (Magalhães et al 1998) and rat ileum (Bezerra et al 2000). This effect was dose related and was achieved at relatively low concentrations. The relaxation of the rabbit duodenum and the decrease in spontaneous mechanical activity induced by terpinen-4-ol were promptly reversed by washing out the compound from the bath, showing functionally that terpinen-4-ol did not cause damage to the tissue contractile apparatus. This finding was similar when the tissues were precontracted with a high-potassium solution.

The muscular α-adrenoceptor has been known to mediate intestinal relaxation ever since the seminal works of Ahlquist (Ahlquist 1948; Ahlquist & Levy 1959) and this phenomenon has been confirmed more recently (Bülbring & Tomita 1987; De Ponti et al 1996). Similarly, the β-adrenoceptor modulates intestinal contractile activity, as indicated by the observation that noradrenaline causes a concentration-dependent relaxation, fully antagonized

Table 1 Maximal intestinal relaxation (R_{max}) induced by terpinen-4-ol (TERP) when applied in the basal tonus in rabbit duodenum and EC50 values (concentration inducing 50% of the maximal response to terpinen-4-ol) in the absence or presence of several pharmacological blockers

Drug	R_{max}	EC50 (95% CI)
TERP + vehicle	173.2 ± 6.4	243.5 (178.0–333.1)
TERP + Phe	162.7 ± 5.9	233.0 (171.0–317.6)
TERP + Phe–PRO	157.6 ± 11.3	244.1 (182.0–327.1)
TERP + vehicle	179.3 ± 11.6	224.5 (164.7–305.8)
TERP + L-NAME	176.9 ± 8.3	243.1 (186.7–316.5)
TERP + ODQ	171.8 ± 10.9	262.4 (196.2–351.0)
TERP + vehicle	160.3 ± 9.0	300.1 (239.8–375.6)
TERP + HEX	165.3 ± 4.6	259.3 (202.2–332.6)
TERP + TTX	155.6 ± 9.3	311.6 (255.6–379.8)
TERP + vehicle	159.8 ± 12.3	222.5 (160.5–308.5)
TERP + charybdo–apamin	182.3 ± 13.8	239.7 (169.5–339.1)
TERP + glibenclamide	176.3 ± 12.4	249.0 (183.4–338.1)
TERP + 4-aminopyridine	165.4 ± 10.3	258.8 (187.3–357.7)
TERP + TEA	184.6 ± 12.8	253.1 (189.5–338.1)

Phe, phentolamine; PRO, propranolol; L-NAME, N^G nitro-L-arginine methyl ester: ODQ, 1*H*-(1,2,4)-oxadiazolo[4,3-a] quinoxalin-1-one; HEX, hexamethonium; TTX, tetrodotoxin: TEA, tetraethylammonium.

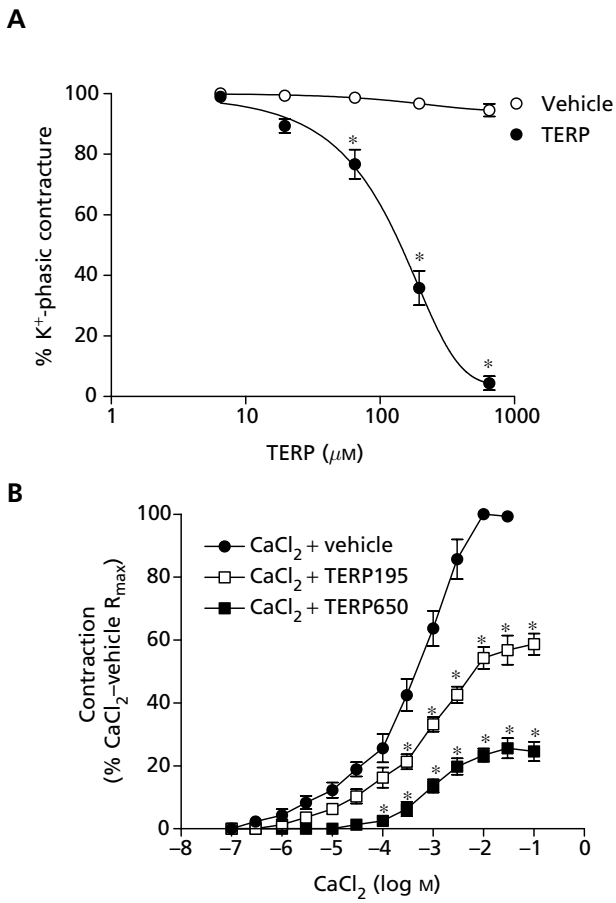

Figure 3 A. The effect of terpinen-4-ol (TERP; 6.5–650 μM) in the phasic component of the contraction in rabbit duodenum elicited by K^+ (60 mM). TERP was incubated for 5 min before K^+ challenge. Vehicle was added isovolumetrically. The data were compared by paired Studen's *t*-test. B. The effect of TERP (195 and 650 μM) on concentration–response curves to calcium chloride (10^{-7} to 10^{-1} M). TERP was incubated for 5 min before the initiation of a curve. The data are expressed as mean ± s.e.m. of 6 experiments. $^*P < 0.05$, TERP vs vehicle; analysis of variance.

by propranolol (Denac et al 1993). Nevertheless, the intestinal relaxation induced by terpinen-4-ol in basal experimental conditions was not blocked by pretreatment of the preparation with an unspecific α-adrenergic blocker, phentolamine, or a combination of phentolamine and the unspecific β-adrenergic blocker, propranolol. Therefore, this effect is related neither directly nor indirectly to activation of adrenergic post-synaptic receptors.

Furthermore, the relaxant activity was not blocked by pretreating the tissues with L-NAME (1 mM), a compound that blocks both the constitutive and inducible isoforms of the enzyme nitric oxide synthase (NOS). Nitric oxide (NO) was demonstrated to be an important inhibitory nonadrenergic-noncholinergic (NANC) neurotransmitter that mediates gastrointestinal motility physiologically in certain pathophysiologic states and is also involved in the laxative action of several compounds (Gaginella et al 1994). Hence, the terpinen-4-ol-induced relaxation is unlike to be mediated by induction of NO release. The participation of the guanylyl cyclase pathway in this intestinal relaxation was ruled out by the finding that ODQ, a selective inhibitor of soluble guanylyl cyclase (Garthwaite et al 1995), was ineffective in blocking this effect.

Additionally, the blockade of nicotinic cholinergic transmission with hexamethonium, in a concentration shown to effectively block the inter-neuron transmission in the autonomic nervous system, including myoenteric plexuses (Goyal & Hirano 1996), was also ineffective in inhibiting terpinen-4-ol-induced intestinal relaxation. Moreover, the classic sodium-channel blocker tetrodotoxin, that ties together, in a highly efficient manner, various parts of the sodium channel and blocks the neuronal action potential (Smythies et al 1971), was equally incapable of blocking terpinen-4-ol-induced relaxation. Therefore, this relaxant activity is not caused by a primary neuronal activity of terpinen-4-ol and is probably myogenic. This hypothesis is reinforced by the similar pattern of terpinen-4-ol-induced relaxation in duodenal preparations precontracted with K^+ (60 mM). Since that potassium concentration displaces the transmembrane resting potential (Em) to $\cong -20$ mV (Grafe et al 1980; Coelho-de-Souza 1997), which inactivates the fast sodium channel and blocks neuronal activity, a neuronal-mediated relaxation is unlikely.

Moreover, the plateau phase of the tonic high potassium induced contraction; the membrane conductance is changed in such a way (Hodgkin & Huxley 1952;

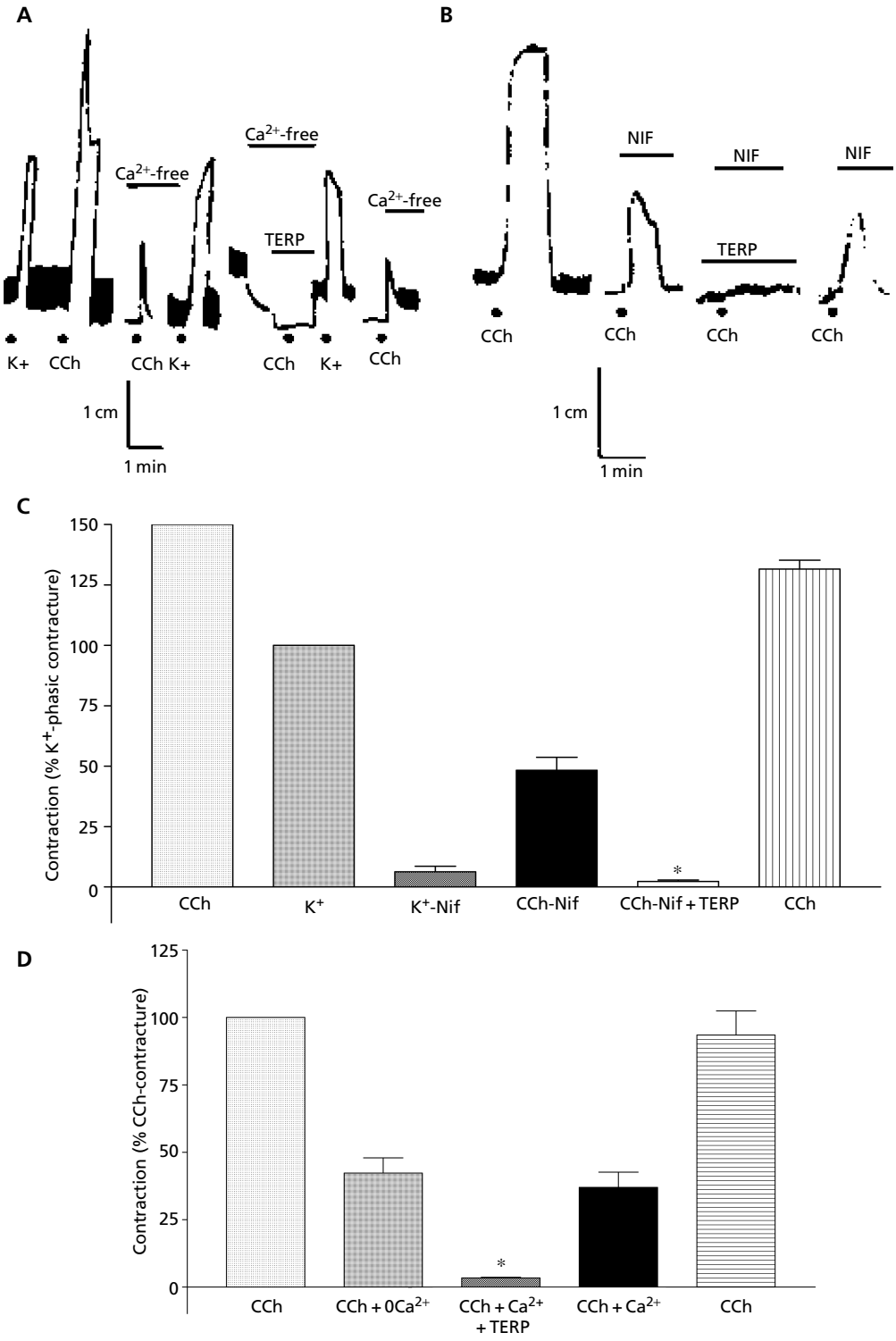

Figure 4 Representative kymographic recordings of the effect of terpinen-4-ol (TERP 195 μM) in contractions evoked by carbachol (CCh) in a calcium-free medium (A) or in the presence of nifedipine (Nif) (B) in rabbit duodenum. C and D. Representation of all experiments for a calcium-free medium and nifedipine pretreatment, respectively. TERP was incubated for 5 min before the carbachol challenge. In the calcium-free protocol, high-potassium was used in a normocalcic solution to replenish the intracellular calcium stores. The data are expressed as mean $\pm$ s.e.m. of 6 experiments. $^*P < 0.05$, vs carbachol in the absence of TERP; analysis of variance followed by Tukey.

Coelho-de-Souza et al 1997; Sousa et al 1997) that no net potassium efflux can be obtained to explain the terpinen-4-ol-induced relaxation. Therefore, it is also unlikely that terpinen-4-ol might relax the duodenum by a K^+-channel opening mechanism. This conclusion is strengthened by the observation that none of the K^+-channel blockers used altered the concentration–response curve for the duodenal relaxation induced by terpinen-4-ol. For instance, the calcium-sensitive K^+-channel blockers charybdotoxin–apamin (Cook & Bryan 1998), the ATP-sensitive K^+-channel blocker glibenclamide (Sturgess et al 1985) or the voltage-dependent K^+-channel blockers 4-aminopyridine or tetraethylammonium (Luzhkov et al 2003; Peuler et al 2004) were ineffective in the blockade of terpinen-4-ol-induced intestinal relaxation.

On the other hand, the phasic contraction induced by K^+ (60 mM) was blocked by terpinen-4-ol in a concentration-related manner. This component of the contraction was shown to be mainly dependent on Ca^{2+} influx via voltage-operated channels (Bolton 1979). Besides, terpinen-4-ol also antagonized Ca^{2+}-induced contractions and this effect was not reversible by increasing calcium concentration in the medium. Therefore, these effects are probably due to an interference with calcium transmembrane transport (Sanders 1989). Alternatively, since the blockade of the Ca^{2+}-induced contractions was not reversible by increasing its concentration in the medium, terpinen-4-ol is probably decreasing the calcium sensitivity of the contratile machinery.

Another important finding is that the component of carbachol (100 μM)-induced contraction that was resistant to nifedipine used in a concentration sufficient to block the contraction induced by a high-potassium (K^+ 60 mM) solution, or that still persisted in a calcium-free medium, was completely blocked by terpinen-4-ol (195 μM). This contraction is mainly due to the release of intracellular bound calcium (Berridge 1983). Therefore, the data presented supports the probability that terpinen-4-ol may either interfere with calcium mobilization from internal stores, with the calcium interaction with proteins (e.g. calmodulin), or in other steps in intracellular calcium signalling as a second messenger. Alternatively, as considered before, terpinen-4-ol could decrease calcium sensitivity of the contractile machinery as demonstrated to be important to the action of other substances (Itoh et al 1994; Rembold 1996).

Conclusion

Terpinen-4-ol is a potent intestinal smooth muscle relaxant and antispasmodic by a direct interaction with intracellular sites that control muscle tonus. These effects are not mediated by interaction with receptors in the membrane or by a potassium-channel opening mechanism. The participation of the NO-GC-GMPc transduction pathway is also discarded as a mechanism for terpinen-4-ol-induced relaxation. A negative interaction with autonomic neurons was also ruled out. Terpinen-4-ol blocks contraction highly dependent on extracellular calcium influx and also blocks contractions elicited mainly by calcium release from internal stores. Terpinen-4-ol has an intracellular mechanism of action, maybe as an antagonist of calcium action as a second messanger. Discovery of the exact mechanism of action of this terpene may reveal new therapeutical targets and deserve further investigation.

Additionally, considering its relatively low toxicity, this pharmacological effect may be useful in conditions of smooth muscle disturbances, such as spasmodic colic and impaction and may also be proved useful in other smooth muscle disturbances, such as bronchial asthma and high blood pressure.

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