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A Ca^{2+} channel blocker-like effect of dehydrocurdione on rodent intestinal and vascular smooth muscle

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Abstract

Effects of dehydrocurdione, a zedoary-derived sesquiterpene, on smooth muscle were investigated by recording the mechanical activity of intestines and aorta from guinea pigs and rats. Dehydrocurdione (0.1–3 mM) induced a sustained relaxation of rat duodenum and inhibited spontaneous motility. Dehydrocurdione (0.1–1 mM) inhibited the contractile response of guinea pig ileum induced by acetylcholine (0.01–10 μM), histamine (0.03–10 μM) and substance P (0.1–30 nM) in a non-competitive manner. Acetylcholine (0.5 μM) elicited a transient contraction followed by a sustained contraction of guinea pig ileum, and dehydrocurdione pretreatment inhibited the sustained component, which depends on Ca^{2+} entry from the extracellular space. The high K^+-induced contraction of rat aortic ring is reported to be blocked by Ca^{2+} channel blockers, while the norepinephrine-induced contraction includes a Ca^{2+} channel blocker-resistant component. Dehydrocurdione (1 mM) blocked the high K^+ (60 mM)-induced contraction of rat aortic ring by 81%, while it inhibited the norepinephrine (1 μM)-induced contraction by only 28%. Dehydrocurdione (1 mM) significantly reduced the high K^+-stimulated increase in cytosolic Ca^{2+} level of Fura-2-loaded mesenteric artery from rats. These results suggest that the inhibitory effects of dehydrocurdione on intestinal and vascular smooth muscle are mediated by blockade of Ca^{2+} entry from the extracellular space.

Keywords: Herbal drug; Sesquiterpene; Ca^{2+} channel blocker; Smooth muscle

1. Introduction

Zedoary (Zedoariae Rhizoma), made from the dried rhisome of *Curcuma zedoaria* Roscoe, is a herbal drug used as a stomachic, that is, a drug to stimulate the secretion of digestive fluid by both olfactory and gustatory reflexes and to promote digestion. Pharmacological effects of zedoary or its water/alcohol extracts have been studied. Zedoary has been shown to increase bile secretion and to inhibit the formation of experimental gastric ulcer in rats and mice (Maeda et al., 1984; Watanabe et al., 1986). Zedoary has been shown to inhibit the intestinal transit of charcoal in mice, to inhibit the contractile responses of the isolated rat duodenum and ileum induced by acetylcholine, histamine and KCl, and to elicit relaxation of the isolated rat duodenum and ileum (Itokawa et al., 1983; Maeda et al., 1984). Ethanol/methanol extracts of zedoary also inhibited rat intestinal contraction stimulated by acetylcholine and barium chloride, and reduced acute experimental gastric ulcers (Itokawa et al., 1983; Watanabe et al., 1986). Zedoary contains many essential oils such as monoterpenes and sesquiterpenes including dehydrocurdione (Fig. 1) (Hikino et al., 1972; Shibuya et al., 1986). Dehydrocurdione is the major component of zedoary cultivated in Yakushima (Japan). We recently reported an anti-inflammatory effect of dehydrocurdione in mice

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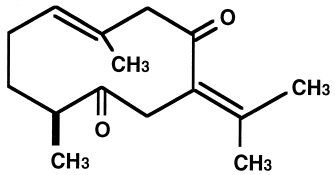

Fig. 1. Chemical structure of dehydrocurdione.

(Yoshioka et al., 1998). In this report, we focused on the ability of dehydrocurdione to inhibit the agonist-induced contraction of intestinal and vascular smooth muscle preparations. Because the contraction of smooth muscle depends on L-type Ca^{2+} channels, the effect of dehydrocurdione on the intracellular Ca^{2+} level was also investigated.

2. Materials and methods

2.1. Chemicals

Dehydrocurdione was extracted from zedoary of Yakushima origin as previously reported (Shibuya et al., 1986), and the purity of the extract was more than 99.9% by gas chromatography–mass spectrometry. Acetylcholine chloride (Ovisot®) was purchased from Daiichi Pharmaceutical (Tokyo, Japan), histamine dihydrochloride from Nacalai tesque (Kyoto, Japan), substance P from Peptide Institute (Osaka, Japan), norepinephrine (NOR-ADRENALIN®) from Sankyo (Tokyo, Japan), hyoscine hydrochloride, verapamil hydrochloride and HEPES from Sigma (St. Louis, MO, USA), and Fura-2-acetoxymethyl ester (Fura-2-AM) from Dojindo (Kumamoto, Japan). Tetrodotoxin was kindly supplied by Sankyo. Other chemicals were purchased from Kanto Chemical (Tokyo, Japan). Dehydrocurdione and verapamil were dissolved in 70% ethanol. Other drugs were dissolved in 0.9% NaCl.

2.2. Animals

Male Hartley guinea pigs (5–9 weeks old) and male Wistar rats (10–14 weeks old) were used. Animals were housed in an air-conditioned room (22 ± 1°C and 55 ± 5% humidity) with a controlled light-dark cycle (6:00–20:00 h light on) and free access to standard chow and tap water. Animals were killed by decapitation to obtain intestine and vascular preparations. The experiments were approved by the Institutional Animal Care Committee.

2.3. Recording of mechanical activity of isolated ileum and duodenum

The ileum at 15 cm proximal to the ileocaecal junction was dissected out from guinea pigs, and the proximal portion of duodenum, except the bulb, was used in rats. Each isolated intestine (2.5 cm in length) was set up in an organ bath containing 5 ml of modified Locke solution (composition in mM: NaCl, 154; KCl, 4.02; $CaCl_2$, 1.36; $MgCl_2$, 0.9; $NaHCO_3$, 2.97; glucose, 5.56; pH, 7.0). Experiments were performed at 37°C with continuous bubbling of air. Mechanical activity of the intestinal segments under a load of 1 g was monitored by an isotonic transducer (Nihonkohden, TD-112S; Tokyo, Japan) connected to a bioelectric amplifier (Nihonkohden, AB-621G) and recorded on a pen recorder (Nihonkohden, WT-645G). After 30 min equilibration, the ileal segments were contracted by 50 mM KCl and then exposed to acetylcholine, histamine or substance P in a cumulative manner to obtain two concentration–response curves for each compound. Then, the effect of pretreatment with dehydrocurdione (final concentration of vehicle was 0.07% ethanol) was examined on the agonist-induced contractions. Each preparation was used for one kind of agonist and for one concentration of dehydrocurdione. Vehicle control experiments were carried out using different preparations for time-matched control. There was a 10-min interval between every drug application.

The effect of dehydrocurdione on the mechanical activity of intestine was studied in isolated rat duodenum. After two test relaxations had been obtained with 2 μM of norepinephrine, duodenal segments were exposed to increasing concentrations of dehydrocurdione at 15-min intervals, during which time the segments regained their original length in fresh medium. In all experiments using both intestinal segments and aortic rings, preparations were washed several times between exposure to KCl/agonists, except with cumulative application.

2.4. Recording of tension of rat aortic ring

Rat thoracic aorta was isolated and cut into rings (2–3 mm in length) and endothelium was removed by rubbing the intimal surface with a cotton-covered glass rod. The endothelium-denuded rings were mounted in an organ bath (Iwashiya Kishimoto Medical Instruments, UC-5; Kyoto, Japan) containing 5 ml of oxygenated (95% O_2–5% CO_2) Krebs–Henseleit solution (composition in mM: NaCl, 118; KCl, 4.7; $CaCl_2$, 2.5; $MgSO_4$, 1.2; KH_2PO_4, 1.2; $NaHCO_3$, 25; glucose, 10; pH, 7.4) maintained at 37°C. The rings were equilibrated for 30 min under a resting tension of 1 g. The tension of the rings was measured by an isometric transducer (Nihonkohden, TB-651T) connected to an isometric amplifier (Nihonkohden, EF-601G) and recorded on a pen recorder (Graphtec, SR6335; Yokohama, Japan). The aortic rings were contracted with 60 mM KCl (high K^+) two times, with washing between applications. The high K^+ or increasing cumulative concentration of norepinephrine was applied to obtain a control contraction. Then the contraction induced by high K^+

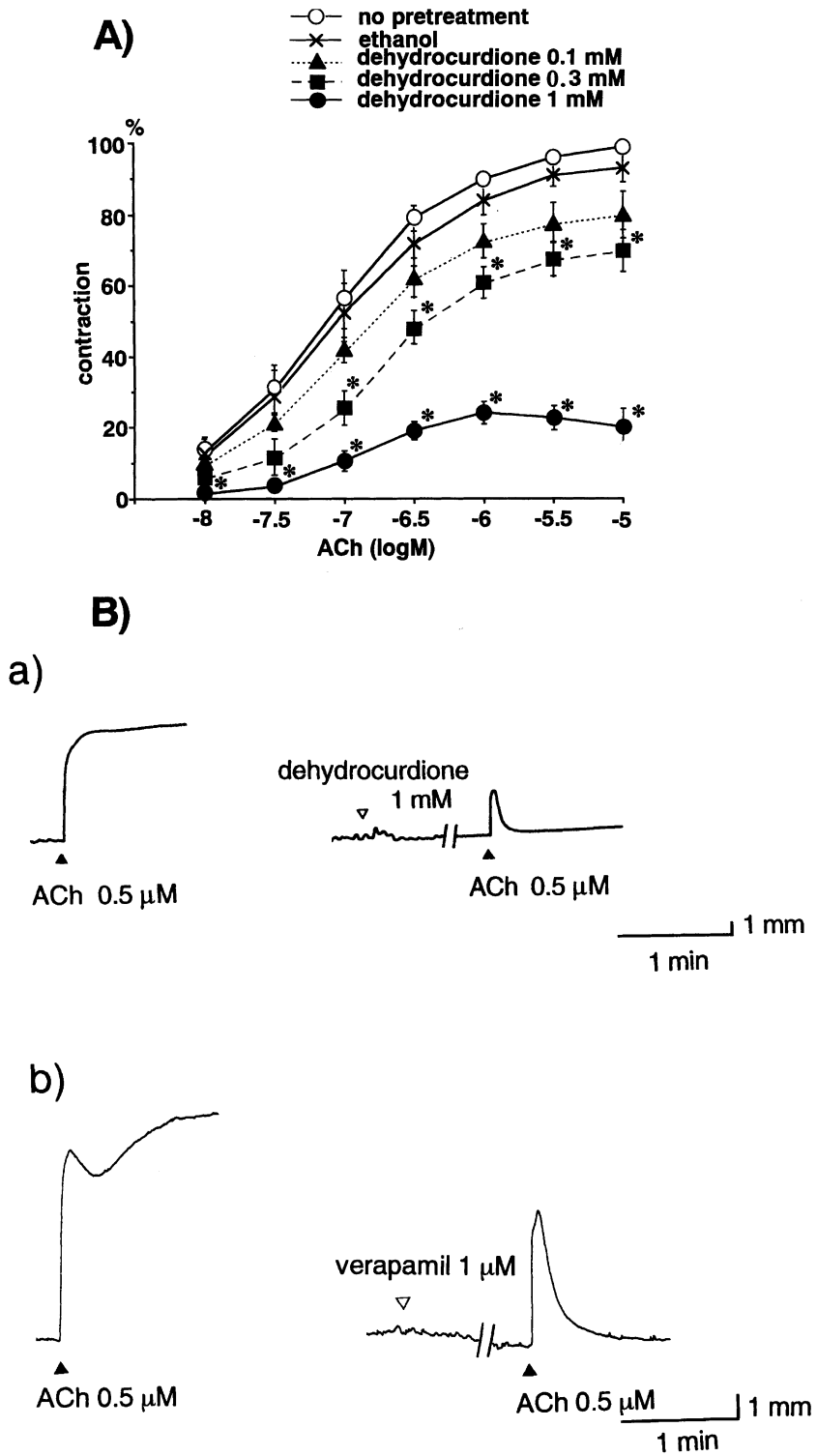

Fig. 2. Effect of dehydrocurdione on acetylcholine-induced contraction of guinea pig ileum. (A) Concentration–response curves of acetylcholine-induced contraction of guinea pig ileum in the presence of 0.1, 0.3 and 1 mM dehydrocurdione or vehicle (0.07% ethanol). Acetylcholine was applied cumulatively and dehydrocurdione was added to the medium 10 min prior to acetylcholine. Values are means ± S.E.M. ($n = 4$, which means number of animals). $^{*}P < 0.05$ vs. non-treated control by unpaired t-test. (B) Effects of 1 mM dehydrocurdione (a) and 1 μM verapamil (b) on 0.5 μM acetylcholine-induced contraction of guinea pig ileum. Preincubation period with verapamil was 2 min in (Bb); otherwise, it was 10 min. The longer preincubation (10 min) with verapamil produced a marked reduction of the peak of the acetylcholine-induced contraction (data not shown). The experiment was repeated four times with similar results.

or norepinephrine was determined in the presence of dehydrocurdione. Time intervals between drug applications were more than 20 min until the tension returned to the baseline. In some preparatons, endothelium removal was checked by the absence of acetylcholine (1 μM)-induced relaxation in the norepinephrine (0.3 μM)-precontracted preparations. The ratio of norepinephrine-induced contraction to high K^+-induced contraction was also examined in each preparation. In our preliminary studies, the ratio norepinephrine-induced contraction/high K^+-induced contraction was about 1.20 and 0.60 in endothelium-denuded and endothelium-intact preparations, respectively.

2.5. Measurement of cytosolic free Ca^{2+} level ($[Ca^{2+}]_i$) of rat mesenteric artery

Cytosolic free Ca^{2+} in isolated vessels was measured by the method of Nakanishi et al. (1997). A strip of mesenteric artery, 5 mm in length, was isolated from the rat and was incubated in HEPES solution (composition in mM: NaCl, 148; KCl, 5; $CaCl_2$, 1.5; $MgCl_2$, 1; glucose, 6; HEPES, 5; pH, 7.4) equibrated with 100% O_2 containing 5 μM Fura-2-AM in the presence of 0.02% cremophor for 2–3 h at room temperature. After dye-loading, the arterial strip was cannulated using two glass pipettes (100–150 μm in tip diameter) placed in an organ bath, and tied to the pipettes with strands of silk suture. The Fura-2-loaded strip was flushed with Krebs solution, and the outer surface of the strip was perfused continuously with warmed (37°C) and oxygenated (95% O_2–5% CO_2) Krebs solution. High K^+ (60 mM) Krebs solution was prepared by replacing NaCl by equimolar KCl. Excitation lights of 340 and 380 nm wavelengths were obtained alternatively using a xenon lamp (450 W), two monochrometors, and a chopper (Spex, Edison, NJ, USA). The intensity of Fura-2 fluorescence at 505 nm during excitation at each wavelength was observed for 1 s at 5-s intervals. After equilibration, the arterial strip was exposed to 60 mM KCl, by changing the perfusion medium to high K^+-Krebs solution. The ratio of Fura-2 fluorescence at 340 nm excitation to that at 380 nm excitation was then calculated and expressed as a percentage, taking the values at rest in normal Krebs solution and in high K^+-Krebs solution to be 0% and 100%, respectively.

2.6. Statistics

All data are expressed as means ± S.E.M. (n is the number of animals studied). The maximal contractile response to the agonist without dehydrocurdione is expressed as 100%. Two-way analysis of variance (ANOVA), followed by unpaired t-test, was used to detect significant differences between control and dehydrocurdione treatment in (Figs. 2, 3 and 6). One-way ANOVA, followed by Bonferroni/Dunn's test, was used to detect differences among concentrations of dehydrocurdione in Fig. 5.

3. Results

3.1. Effect of dehydrocurdione on agonist-induced contractions of guinea pig ileum

Cumulative application of acetylcholine (0.01–10 μM) induced concentration-dependent contraction of the guinea pig ileum, and pretreatment with dehydrocurdione (0.1–1 mM) inhibited the acetylcholine-induced contraction (Fig. 2A). There was a significant difference in the contraction

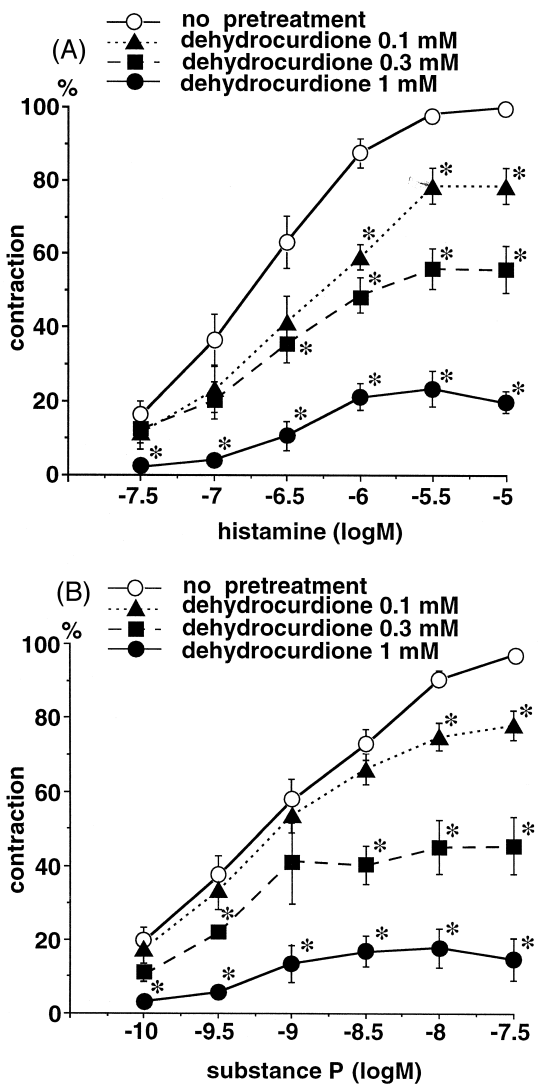

Fig. 3. Concentration–response curves of histamine- (A) and substance P- (B) induced contractions of guinea pig ileum in the absence and presence of 0.1, 0.3 and 1 mM dehydrocurdione. Histamine and substance P were applied cumulatively and dehydrocurdione was added to the medium 10 min prior to the agonists. Values are means ± S.E.M. ($n = 4$ each). * $P < 0.05$ vs. control (no pretreatment) by unpaired t-test.

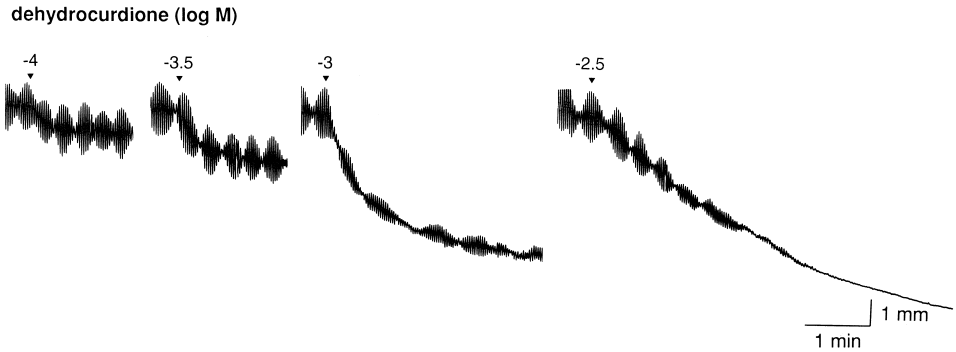

Fig. 4. Effects of dehydrocurdione on the mechanical activity of isolated rat duodenum. Increasing concentrations of dehydrocurdione were applied at 15-min intervals, the time which was required for washing the strips and recovery of tone. Similar concentration-dependent relaxations were observed in four preparations from different animals.

between ethanol control and the tissue treated with 0.1 mM dehydrocurdione ($P < 0.01$ by ANOVA). As shown in Fig. 2A, dehydrocurdione reduced the maximal response to acetylcholine concentration dependently, indicating that

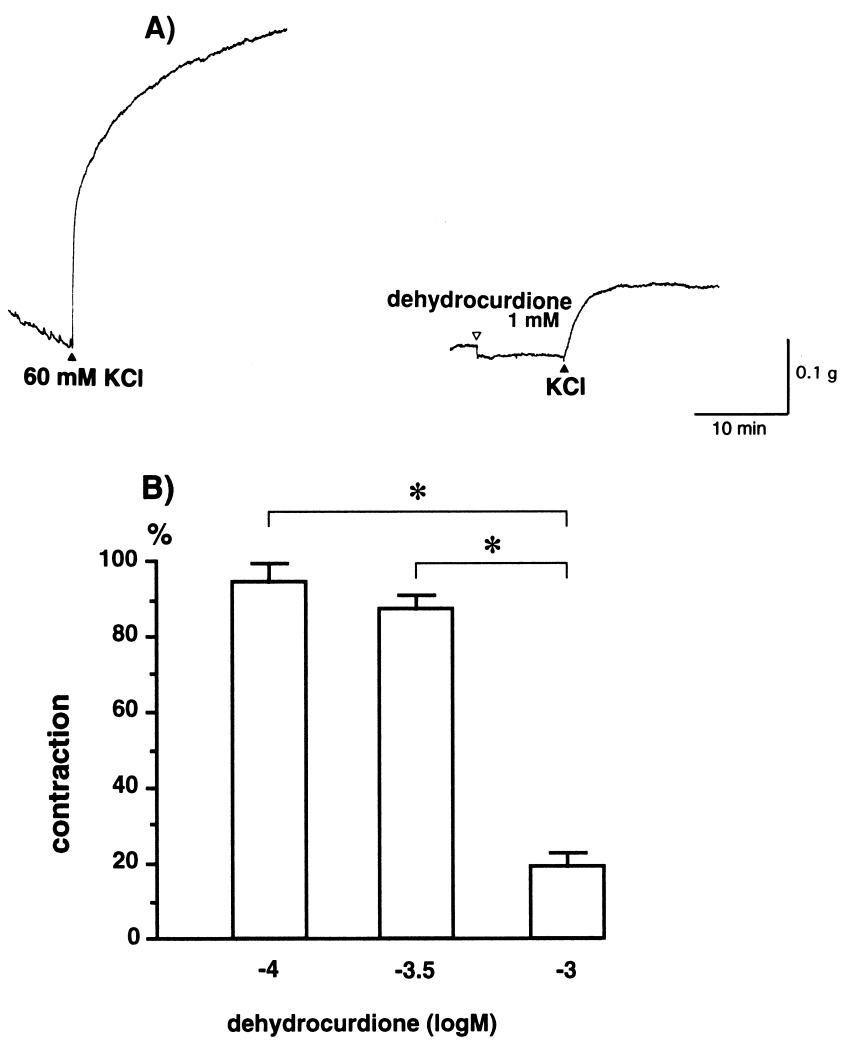

Fig. 5. Effect of dehydrocurdione on high K^+-induced contraction of rat aortic ring. (A) Typical recordings of high K^+-induced contraction in the presence and absence of dehydrocurdione. Dehydrocurdione was added to the medium 10 min prior to 60 mM KCl. (B) Effect of increasing concentrations of dehydrocurdione on high K^+-induced contraction. The maximal contraction during 16-min incubation period with high K^+ in the absence of dehydrocurdione was determined as 100%. Values are means ± S.E.M. ($n = 4$ each). Difference among concentrations of dehydrocurdione was significant by one-way ANOVA ($P < 0.001$) followed by Bonferroni/Dunn's test ($^* P < 0.05$).

the inhibitory action of dehydrocurdione on acetylcholine was noncompetitive antagonism. The pD'_2 (negative logarithm of molar concentration of noncompetitive antagonist to induce 50% inhibition of the maximal response to agonist) of dehydrocurdione for acetylcholine-induced contraction was 3.32.

Acetylcholine (0.5 μM) induced a transient followed by sustained contraction in Locke's solution, although the two components usually were not clearly defined (Fig. 2Ba and b). The response to acetylcholine was converted to a transient contraction without a sustained component in the presence of 1 mM dehydrocurdione or 1 μM verapamil, an L-type Ca^{2+} channel blocker (Fig. 2B). This indicates that the sustained component of the acetylcholine-induced contraction is sensitive to Ca^{2+} channel blockers and that dehydrocurdione has a Ca^{2+} channel blocker-like property.

Histamine (0.03–10 μM) and substance P (0.1–30 nM) also induced concentration-dependent contractions of guinea pig ileum, which were again inhibited by dehydrocurdione (Fig. 3). Pretreatment with 0.07% ethanol, the vehicle for dehydrocurdione, did not change the contractile responses of ileum to histamine and substance P (not shown). The difference in contraction between no-treatment control and tissue treated with 0.1 mM dehydrocurdione was significant for histamine- and substance P-induced contractions ($P < 0.001$ by ANOVA). As shown in Fig. 3A and B, the maximal responses to the agonists were decreased in the presence of 0.1–1 mM dehydrocurdione, indicating noncompetitive antagonism. The pD'_2 values of dehydrocurdione were calculated as 3.42 and 3.59 for contractions induced by histamine and substance P, respectively.

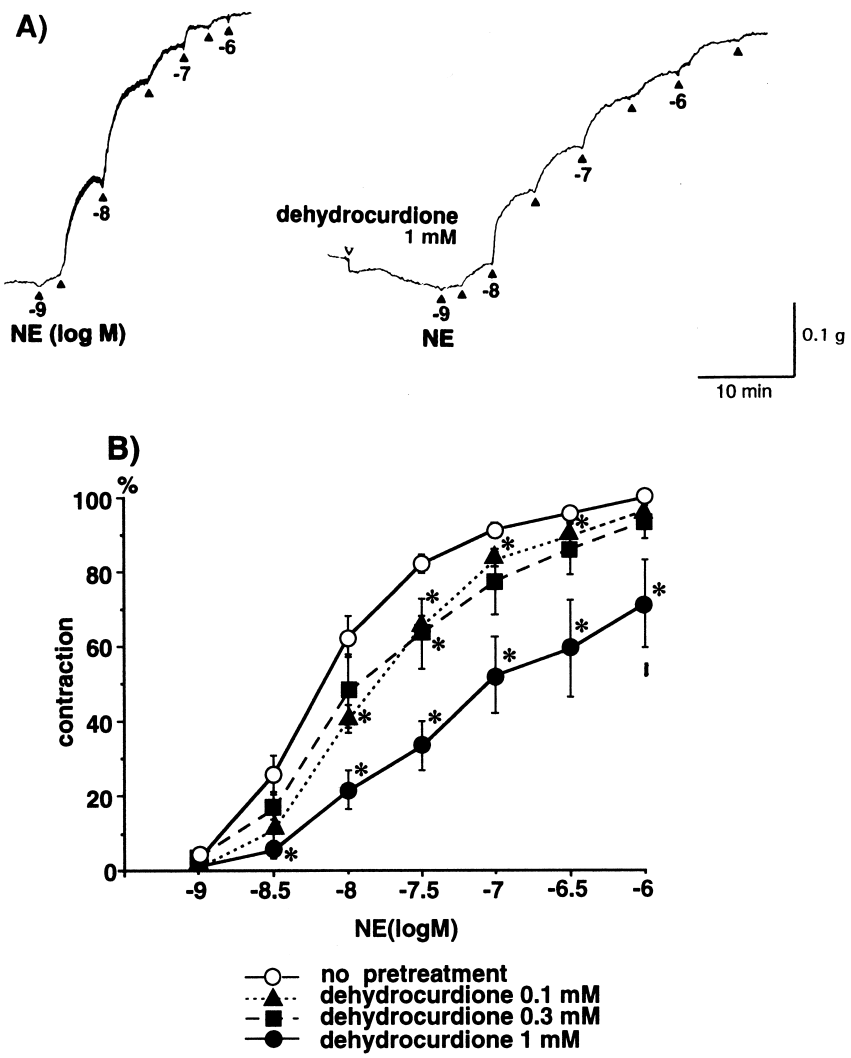

Fig. 6. Effect of dehydrocurdione on norepinephrine-induced contraction of rat aortic ring. (A) A typical recording of norepinephrine-induced contraction in the presence and absence of 1 mM dehydrocurdione. Dehydrocurdione was added to the medium 10 min prior to cumulative addition of norepinephrine. (B) Concentration–response curves of norepinephrine-induced contraction of rat aorta in the presence of 0.1, 0.3 and 1 mM dehydrocurdione. Values are means ± S.E.M. ($n = 4$ each). Difference between control and 0.3 mM (or 1 mM) dehydrocurdione-treated group was significant by two-way ANOVA ($P < 0.001$) followed by unpaired t-test ($^{*}P < 0.05$ vs. non-treated control).

All contractions elicited by acetylcholine (1 μM), histamine (1 μM) and substance P (10 nM) were not influenced by tetrodotoxin (1 μM) pretreatment (data not shown), indicating these contractions were myogenic responses.

3.2. Effects of dehydrocurdione on the mechanical activity of isolated rat duodenum

Because rat duodenum is much more sensitive to relaxants than guinea pig ileum and has substantial spontaneous motility, we studied the effect of dehydrocurdione on the mechanical activity of rat duodenum. Dehydrocurdione (0.1–3 mM) induced a sustained relaxation of rat duodenum in a concentration-dependent manner, and in higher concentrations (1–3 mM) it inhibited the spontaneous motility of the duodenum (Fig. 4). Neither tetrodotoxin (1 μM) nor hyoscine (0.25 μM) blocked the spontaneous motility of the duodenum (not shown), indicating that this motility was of myogenic origin. The dehydrocurdione-induced relaxation of rat duodenum was not influenced by tetrodotoxin pretreatment (not shown), suggesting the direct relaxant effect of dehydrocurdione on smooth muscle cells.

3.3. Effects of dehydrocurdione on high K^+- and norepinephrine-induced contractions of rat aortic ring

Ca^{2+} channel blockers are reported to extensively block high K^+-induced but to partially block norepinephrine-induced contraction of rat aorta (Ozaki et al., 1990; Karaki et al., 1997). To investigate the properties of dehydrocurdione as a Ca^{2+} blocker in the vasculature, we examined the effect of dehydrocurdione on contraction of rat aortic rings induced by 60 mM KCl (high K^+), which induces maximal contractile responses, and by cumulative application of norepinephrine. Dehydrocurdione concentration dependently inhibited the high K^+- and norepinephrine-induced contraction of rat aorta (Figs. 5B and 6B). The inhibitory effect of dehydrocurdione (1 mM), however, was much greater on the high K^+-induced contraction (81%, Fig. 5) than on the norepinephrine (1 μM)-induced contraction (28%, Fig. 6). The pD'_2 value of dehydrocurdione for the high K^+-induced contraction of rat aorta was 3.23.

3.4. Effects of dehydrocurdione on high K^+-induced increase in $[Ca^{2+}]_i$ of rat mesenteric artery

Cytosolic free Ca^{2+} in rat mesenteric artery was monitored using Fura-2 fluorescence. High K^+ increased $[Ca^{2+}]_i$ of mesenteric artery, and the increase in $[Ca^{2+}]_i$ was reduced by dehydrocurdione in a concentration-dependent manner (Fig. 7). The inhibition induced by 1 mM dehydrocurdione was 58 ± 3.7% ($n = 5$).

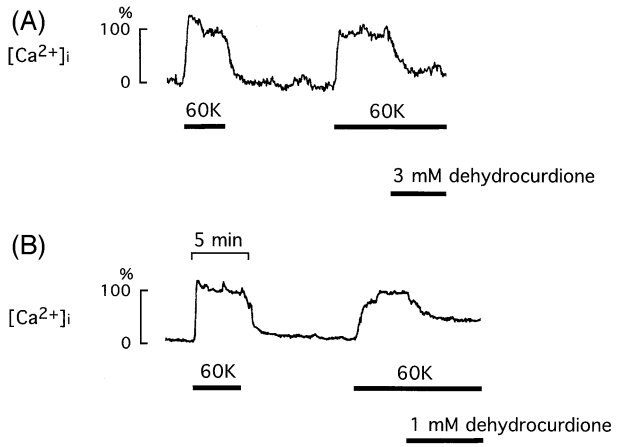

Fig. 7. Effect of dehydrocurdione (3 mM in (A) and 1 mM in (B)) on $[Ca^{2+}]_i$ of rat mesenteric artery stimulated by 60 mM KCl. The Fura-2 fluorescence ratio (F340/F380) at rest and after addition of 60 mM K^+ was considered to be 0% and 100%, respectively. When the Fura-2 response to 60 mM K^+ (60 K) reached a plateau, perfusion medium was changed to dehydrocurdione-containing high K^+-Krebs solution. Ethanol (0.07%) did not affect the high-K^+-induced $[Ca^{2+}]_i$ elevation in mesenteric artery (not shown).

4. Discussion

Zedoary is used as a stomachic, and water/alcohol extracts of zedoary have inhibitory effects on the gastrointestinal tract in rodents (Itokawa et al., 1983; Maeda et al., 1984; Watanabe et al., 1986). It is not known how this inhibitory effect of water/alcohol extracts of zedoary has a role in the stomachic effect of zedoary. In this study, dehydrocurdione, a major sesquiterpene in zedoary, inhibited contractions of guinea pig ileum stimulated by acetylcholine, histamine and substance P in a non-competitive manner. Dehydrocurdione relaxed the rat duodenum from the resting level and also decreased the spontaneous motility of the rat duodenum. The non-specific inhibitory effect of dehydrocurdione on agonist-induced contractions of guinea pig ileum is consistent with the results of previous studies using the water-soluble fraction (Maeda et al., 1986) and ethanol/methanol extracts (Itokawa et al., 1983; Watanabe et al., 1986) of zedoary in rat and guinea pig intestine. Inhibition of the resting tonus and the spontaneous motility of the duodenum by dehydrocurdione is again consistent with previous results obtained with the water-soluble fraction of Zedoary (Maeda et al., 1986).

Contractions of smooth muscle are regulated by the intracellular free Ca^{2+} concentration. Ca^{2+} originates from both the intracellular Ca^{2+} store, the sarcoplasmic reticulum, and from influx from the extracellular space (Somlyo and Somlyo 1994; Karaki et al., 1997). The contractile

response of smooth muscles including guinea pig ileum to agonists is composed of an initial transient contraction followed by a sustained contraction, which depend on release from the Ca^{2+} store and Ca^{2+} influx, respectively (Macara and Giao-T. Rico, 1992; Somlyo and Somlyo, 1994; Karaki et al., 1997). Only the sustained (but not the transient) part of the contraction induced by acetylcholine (0.5 μM) was blocked by dehydrocurdione and verapamil. This result indicates that dehydrocurdione blocks the entry of Ca^{2+} from the extracellular space in guinea pig ileum, as do other Ca^{2+} blockers. In smooth muscle cells, the voltage-dependent L-type Ca^{2+} channel is considered to be a major Ca^{2+} influx pathway (Karaki et al., 1997), and this type of Ca^{2+} channel does not exist in enteric neurons (Surprenant, 1994).

The relaxation of rat duodenum by dehydrocurdione supports the idea that the effect is related to the Ca^{2+} channel, because inhibition of Ca^{2+} influx by Ca^{2+} channel blockers can reduce $[Ca^{2+}]_i$ and cause relaxation of smooth muscle (Somlyo and Somlyo, 1994; Karaki et al., 1997). A new Ca^{2+} blocker CAF603, a sesqueterpene isolated from *Trichoderma virens*, similarly decreased the tonus of guinea pig duodenum and inhibited spontaneous activity (Suarez-Kurtz et al., 1997).

We provided other evidence for the Ca^{2+} channel blocker-like property of dehydrocurdione in experiments with rat aorta. In the rat aorta, it has been shown that the high K^+-induced contraction depends on an increase in $[Ca^{2+}]_i$ through the L-type Ca^{2+} channel, which is activated by depolarization, whereas the norepinephrine-induced contraction depends on increases in both $[Ca^{2+}]_i$ and Ca^{2+} sensitivity of contractile elements (Karaki et al., 1997). This was substantiated by the experiments in which the high K^+-induced contraction was almost completely blocked by a Ca^{2+} channel blocker, such as verapamil, whereas the norepinephrine-induced contraction was only partially blocked by verapamil (Sato et al., 1988; Ozaki et al., 1990; Karaki et al., 1997). The inhibitiory profiles of dehydrocurdione on K^+- and norepinephrine-induced contractions of rat aortic ring in this study were similar to the effect of verapamil on rat aorta in previous reports (Sato et al., 1988; Ozaki et al., 1990).

Overall, the effects of dehydrocurdione on smooth muscle preparations in this study suggest that this drug acts on smooth muscle cells as a Ca^{2+} channel blocker and induces non-specific inhibition of smooth muscle contraction. The experiment in which $[Ca^{2+}]_i$ was measured in the rat mesenteric arteries provided direct evidence for the ability of dehydrocurdione to act as a Ca^{2+} channel blocker. Dehydrocurdione decreased $[Ca^{2+}]_i$ pre-elevated by high K^+. The increase in $[Ca^{2+}]_i$ by high K^+ has been shown to result from Ca^{2+} entry through L-type Ca^{2+} channels in consequence of membrane depolarization (Karaki et al., 1997).

Dehydrocurdione has a relatively low potency compared with other Ca^{2+} blockers. However, its chemical structure is different from that of any previous Ca^{2+} blockers, such as dehydropyridines, benzothiazepines and phenylalkylamines. Therefore, dehydrocurdione or its analogues may be used as a lead drug for a new class of Ca^{2+} channel blocker.

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