ROLE OF AN INTRACELLULAR Ca STORE IN TREPIBUTONE (AA-149)-INDUCED RELAXATION OF THE DEPOLARIZED SMOOTH MUSCLE OF THE GUINEA-PIG TAENIA COLI

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Abstract—The depolarized smooth muscle of guinea pig taenia coli suspended in Ca-free, K-rich Krebs solution showed a tension development with the addition of CaCl₂ (Ca contracture) and the increased tension returned to the original level by rinsing with the solution. Application of carbachol (CCh) to this preparation caused an increase in tension despite the absence of Ca in the medium (CCh contraction), indicating a Ca release from intracellular stores. Trepibutone, a newly synthesized smooth muscle relaxant, given before CaCl₂ prevented the Ca contracture and reduced the increased tension when given after CaCl₂. The CCh contraction was enhanced after inhibition of the Ca contracture by trepibutone. D-600, a Ca antagonist, inhibited the Ca contracture and CCh contraction. The inhibitory effect of trepibutone on the Ca contracture was markedly reduced in the presence of CCh, whereas that of D-600 was not affected. These results suggest that trepibutone, unlike D-600, does not have an inhibitory action on Ca influx but rather an accelerating action on the uptake of intracellular Ca into the Ca stores sensitive to CCh.

We have already demonstrated that trepibutone [3-(2,4,5-triethoxybenzoyl)propionic acid: AA-149] causes a relaxation of the sphincter of Oddi in dogs and in rabbits through a direct action on smooth muscle (1, 2). The spasmytic effect apparently differs from that of papaverine, because trepibutone (10⁻⁵–10⁻³ M) had no effects on the contractile response to ACh in the isolated sphincter of Oddi and duodenum of rabbits (2). The tone of the smooth muscle depends on an intracellular ionized Ca concentration which is regulated by both the extracellular Ca concentrations and intracellular Ca stores. A simple technique developed by Ohashi et al. (3, 4) has enabled studies on the effects of a drug on Ca movement by recording the isometric tension development of the depolarized smooth muscle of the guinea-pig taenia coli. These workers found that the taenia coli possesses an intracellular Ca store, from which Ca is released by carbachol (CCh). We used this technique to investigate the role of intracellular Ca stores in trepibutone-induced relaxation of the smooth muscle and the obtained data were compared with findings in the case of D-600, an organic Ca antagonist.

MATERIALS AND METHODS
Preparations: Hartley guinea-pigs of either sex weighing 350–450 g were exsanguinated and strips of the taenia coli were prepared and immersed in a Ca-free, K-rich Krebs
Solutions and measurements of the mechanical response: The smooth muscle preparation of taenia coli, about 20 mm in length, was suspended in an organ bath containing 20 ml of Ca-free, K-rich Krebs solution of the following composition (mM): KCl, 126.6; KHCO₃, 15.5; KH₂PO₄, 1.2; MgCl₂, 1.2 and glucose, 11.5. The bathing solutions were kept at 20°C and continuously bubbled with 95% O₂ and 5% CO₂. After an equilibration period of 30 min, the resting tension was adjusted to 0.5 g. The contractile tension of the preparation was measured isometrically using a force displacement transducer (Nihon Koden, SB1T) and recorded on a polygraph (Nihon Koden, RM 85).

Agents: The following agents were used: carbachol (CCh, Tokyo Kasei), α-isopropyl-α-(N-methyl-N-homoveratryl)-γ-amino-propyl)-3,4,5-trimethoxyphenylacetonitril hydrochloride (D-600, synthesized in Takeda Labs.) and trepibutone [3-(2,4,5-triethoxybenzoyl)propionic acid; AA-149, synthesized in Takeda Labs.]. Trepibutone was dissolved in 1N NaOH and the pH was adjusted to 7.0 with 1N HCl.

Statistical evaluation: The values obtained were expressed as mean values and their standard error. The significance of differences of mean values between the two groups was assessed using Student's t-test for unpaired values.

RESULTS

Effects of trepibutone and D-600 on the Ca contracture: The smooth muscle preparation was suspended in Ca-free, K-rich Krebs solution for at least 30 min to obtain a complete relaxation. The preparation was then exposed to various concentrations of CaCl₂ (0.16–2.5×10⁻³ M), thus allowing the tension to develop at each concentration of Ca. Before each addition of Ca, the preparation was relaxed by rinsing with the Ca-free, K-rich Krebs solution. The tension increased with latencies of 3–30 sec after the addition of Ca and attained a maximum
level within several minutes (Fig. 1a). The maximum tension was maintained for more than 10 min. This Ca-induced response was termed "Ca contracture". It was found that by increasing Ca concentration, the maximum tension and rate of tension development were increased and the latency was shortened (Fig. 1b).

Under Ca-free conditions, trepibutone (10^{-5}–10^{-3} M) and D-600 (10^{-7}–10^{-5} M) produced no change in the tension of the preparation. However, these agents did suppress the Ca contracture in a concentration-dependent manner, when CaCl_2 (1.25 \times 10^{-3} M) was added five min later. Figure 2a shows the inhibitory effects of trepibutone (10^{-4} M) and D-600 (10^{-6} M) on the Ca contracture. The inhibitory potency of trepibutone was approximately one-hundredth that of D-600 (Fig. 2b). In addition, the effect of trepibutone on the pattern of Ca contracture differed markedly from that of D-600. D-600 delayed the onset of the Ca contracture and decreased the rate of development of the tension (Fig. 2c and d, left). Trepibutone depressed the maximum tension with no change of the latency and with no consistent changes in the rate of tension development (Fig. 2c and d, right). The pattern of the Ca contracture in the presence of D-600 was similar to that obtained when the concentration of external Ca was decreased, as shown in Fig. 1.

When trepibutone (10^{-5}–10^{-3} M) or D-600 (10^{-7}–10^{-5} M) was applied five min after the addition of CaCl_2 (1.25 \times 10^{-3} M), the tension increased by Ca was consistently decreased in a concentration-dependent manner. Figure 3a shows the reducing effects of trepibutone (10^{-4} M) and D-600 (10^{-6} M) on the maximum tension of the Ca contracture, and the magnitude of the reduction measured 10 min later was 70±3% (n=5) and 67±4% (n=4), respectively. The concentration-response relationship of both compounds is summarized in Fig. 3b.

Effect of trepibutone and D-600 on the CCh contraction: The preparation suspended in the Ca-free, K-rich Krebs solution showed
Fig. 3. Effects of trepibutone (AA-149) and D-600 on the increased tension of the depolarized taenia coli in the Ca contracture. a) Reduction of the increased tension by trepibutone (10^{-4} M) and D-600 (10^{-6} M) added to the bath 5 min after addition of 1.25 \times 10^{-3} M CaCl_2. Each tension was compared to the tension obtained 5 min after addition of 1.25 \times 10^{-3} M CaCl_2 as 100%. b) Drug concentration-decrease in tension curve. Trepibutone or D-600 was given 5 min after addition of Ca, and the reduction of tension induced by the drugs was measured 10 min later. ***P<0.001 vs control.

Fig. 4. The CCh contraction in the depolarized taenia coli pretreated with various concentrations of Ca. a) Increase in tension of the depolarized taenia coli by 10^{-5} M CCh. W: the preparation was washed 3 times with the Ca-free, K-rich Krebs solution. b) Peak tension of the CCh contraction in the depolarized taenia coli pretreated with various concentrations of CaCl_2. Mean value of the peak tension of the CCh contraction in the depolarized taenia coli pretreatment with 1.25 \times 10^{-3} M CaCl_2 is expressed as 100%. Each point represents mean±S.E.M. of 5 experiments.

Fig. 4b shows the response of the preparation to 10^{-5} M CCh. It can be seen that the response occurred immediately after application of CCh and subsided within 10 min. However, this preparation usually lost the capacity to respond with subsequent application of CCh (Fig. 4a). If the preparation was then exposed to various concentrations of Ca for 5 min, the tension was increased (the Ca contracture). The increased tension of the Ca contracture returned to original levels by exposing the preparation to Ca-free solution, but did regain the capacity to produce a contractile response to CCh, as was also observed by Ohashi et al. (3, 4). This CCh-induced response was termed “CCh contraction”. As shown in Fig. 4b, the amplitude of the CCh contraction increased in proportion to the concentrations of CaCl_2 (0.16–2.5 \times 10^{-3} M) added during the Ca contracture. The most plausible explanation for these results is that the smooth muscle possesses intracellular Ca stores where Ca ions are accumulated or bound and CCh ions are bound.
thereby causes a contraction by releasing Ca from the site. When trepibutone (10^{-5}–10^{-3} M) or D-600 (10^{-7}–10^{-5} M) was given 5 min before the exposure to CaCl\(_2\) (1.25 \times 10^{-3} M), the Ca contracture was inhibited (Fig. 5). However, the amplitude of CCh contraction obtained after rinsing with the Ca-free solution differed in both groups. D-600 produced a decrease in the amplitude of the CCh contraction in proportion to the reduction of the Ca contracture. However, trepibutone enhanced the CCh contraction significantly at 10^{-4} M, despite the obvious inhibition of the Ca contracture.

To assess the role of intracellular Ca stores in the inhibitory effects of trepibutone and D-600 on the Ca contracture, the effects were compared either in the absence or presence of high concentrations of CCh (10^{-3} M). When CaCl\(_2\) (1.25 \times 10^{-3} M) was...
added in the presence of CCh, the contractile response reached a peak tension with the same time course as seen in the absence of CCh (Fig. 6a). The peak tension was slightly higher than the maximum tension in the absence of CCh, but gradually declined to 67±6% at 10 min later in the presence of CCh, as shown in Fig. 6a. When CCh (10^-3 M) was simultaneously applied with trepibutone (10^-5–10^-3 M) or D-600 (10^-7–10^-5 M) 5 min before the addition of CaCl2, the inhibitory effect of D-600 on the Ca contracture was enhanced, whereas that of trepibutone was reduced (Fig. 6b).

**DISCUSSION**

Trepibutone produced concentration-dependent reductions in the Ca contracture in Ca-free, K-rich Krebs solution. Recently, Kishimoto et al. (5) reported that ouabain produced a relaxation of the depolarized smooth muscle of guinea-pig taenia coli by a mechanism different from those of 2,4-dinitrophenol (DNP) and verapamil, i.e., by an accumulation of cellular Na followed by an inhibition of Ca influx. On the other hand, it has been suggested that DNP and verapamil produced the relaxation of smooth muscle by an acceleration of Ca efflux induced by a severe metabolic inhibition (6) and by a competitive inhibition of Ca influx (7), respectively. In this study, a Na-free solution containing 143.3 mM K was used to depolarize the smooth muscle membrane. Therefore, mechanisms related to relaxation of trepibutone would differ from those in the case of ouabain, and possible changes in membrane potential can be excluded. In addition, it seems unlikely that trepibutone relaxes the smooth muscle by the severe metabolic inhibition, because the toxicity of trepibutone, unlike DNP, was very low, i.e., the LD50 was over 2,000 mg/kg.p.o. in rats (unpublished data). Consequently, our interest was focused on whether trepibutone, like verapamil, has competitive inhibitory actions on the influx of Ca. In this experiment, we compared the effect of trepibutone on Ca movement with that of a methoxy-derivative of verapamil (D-600) which was reported by Mayer et al. (8) to have an inhibitory action on the Ca influx in the smooth muscle of the guinea-pig taenia coli. We found that trepibutone differed from D-600 in two major respects. First, trepibutone had a different effect on the pattern of the Ca contracture. The pattern of Ca contracture i.e., the latency, rate of tension development and maximum tension, was closely dependent upon the concentration of Ca added, and the pattern of a given concentration of Ca in the presence of D-600 could be readily mimicked using a lower concentration of Ca. These results parallel the findings of Mayer et al. (8). However, trepibutone reduced the maximum tension of the Ca contracture without any appreciable change in the latency and only a slight decrease in rate of tension development. These results suggest that trepibutone depressed the tension development by a mechanism other than a competitive inhibition against Ca influx.

Secondly, differences were observed in the amplitude of CCh contraction obtained after the inhibition of the Ca contracture by both compounds. The amplitude of the CCh contraction was closely dependent upon the concentration of Ca added in the Ca contracture and reduced when the Ca contracture was prevented by D-600, as observed by Ohashi et al. (3, 4). These results can be explained under the assumption that some fraction of the Ca across the membrane during the Ca contracture is taken upon into the intracellular Ca store, and that CCh would cause contraction by releasing these Ca ions. This postulation is supported by the data that CCh stimulated a release of 45Ca into the medium from the depolarized smooth muscle cell of the guinea-pig taenia.
coli (9). If trepibutone did prevent tension development of the Ca contracture by inhibiting the Ca influx, the amplitude of the CCh contraction would be reduced. However, it was not reduced, rather there was a significant enhancement at 10^{-4} M. These results support the suggestion that trepibutone does not have an inhibitory action on Ca influx. In addition, trepibutone probably prevents the Ca contracture by decreasing the amount of free Ca available to the contractile elements by accelerating the uptake of Ca into the store. This view is supported by the fact that the inhibitory effect of trepibutone on the Ca contracture was significantly reduced in the presence of high concentrations of CCh, to the extent that the Ca storage would probably be depleted. However, the enhancement of the CCh contraction by trepibutone was observed at the concentration of 10^{-4} M, and the degree of enhancement was about 20%, despite 70% inhibition of the Ca contracture. Therefore, additional mechanisms may be involved.

It is concluded that trepibutone, unlike D-600, does not have a competitive inhibitory action on Ca influx in the depolarized smooth muscle of the guinea-pig taenia coli, but does have an accelerating action on the uptake of Ca into the intracellular Ca store sensitive to CCh. The latter effect may explain in part the relaxation mechanism of trepibutone on the smooth muscle.

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