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Effects of Cyclobuxine E on Two Distinct Types of Potassium-Activated Calcium Channels in an Intestinal Smooth Muscle

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ABSTRACT

For several years, we investigated the pharmacological action of several substances isolated from *Buxus microphylla* var *koreana* Nakai, which had been used as folk remedies of malaria and venereal disease. Cyclobuxine D ($C_{25}H_{42}ON_2$), a steroidal alkaloid, exerted an antiinflammatory action, hypotensive and bradycardic effects in rats.

In the present study, we isolated alkaloid from the acetone-insoluble fraction of the strong bases of this plants. This alkaloid ($C_{24}H_{38}ON_2$) was identified as a steroidal alkaloid contained a cyclopropane ring by physical and chemical methods. It is a derivative of cyclobuxine D and named cyclobuxine E.

We examined the effect of cyclobuxine E on the contractile response induced by acetylcholine and two distinct types of potassium-activated calcium channels in an intestinal smooth muscle of the rat. Cyclobuxine E inhibited significantly the Ach-induced contraction.

The isolated longitudinal muscle from the rat duodenum was immersed calcium-depleted potassium depolarizing solution. Ten minutes after, 1.8 mM $CaCl_2$ was added to muscle bath and elicited a biphasic increase in muscle tension. Cyclobuxine E produced an appreciable inhibition of both components of the mechanical response. In addition, Cyclobuxine E introduced at a point when the tonic response had reached its maximum level, caused the muscle to exhibit a rapid loss of tension. Based on these experimental results, we proposed the possibility that the inhibitory action of cyclobuxine E on the isolated rat duodenum may be due to inhibiting the transmembrane fluxes of calcium ion in potassium-activated calcium channels.

Key Words: Cyclobuxine E, Acetylcholine, Biphasic response, Potassium-activated calcium channels

INTRODUCTION

Smooth muscle contraction is closely related to levels of intracellular free calcium. Intracellular free calcium levels are regulated by transport via calcium channel (Bullbrign & Tomita, 1970; Burgen, 1970; Bolton, 1979; Kostyuk, 1980), calcium pump (Schatzmann, 1975), Na^+-Ca^{++}-exchange site (Blaustein, 1974; Grover *er al.,* 1983), uptake and release from sarcoplasmic reticulum, release from the binding site of cell membrane (Carafol & Crompton, 1978) and phosphatidylinositol turnover (Michell, 1975; Michell *er al.,* 1976). It has been shown that some drugs relaxed the smooth muscle due to a reduction of intracellular calcium concentration as a consequency of the inhibition of calcium influx through voltage or receptor-dependent calcium channels of the cell membrane.

Kwon *et al.,* (1988) reported that cyclobuxine D, extracted from *Buxus microphylla* var koreana Nakai, inhibited the contractile response-induced by drugs in the isolated smooth muscle from the rabbit intestine and rat uterus. In this study, we extracted cyclobuxine E (derivative of cyclobuxine D) from *Buxus microphylla* var koreana Nakai and investigated the effect of cyclobuxine E in the contractile response-elicited by acetylcholine in duodenal smooth muscle of the rat. In order to analyse the inhibitory action of cyclobuxine E on the smooth muscle. We examined the inhibitory action of cyclobuxine E against the contractile responses of the high potassium-depolarized rat

duodenum to calcium.

MATERIALS AND METHODS

Extraction and identification of cyclobuxine E

Dried leaves of *Buxus microphylla* var *koreana* Nakai (7.1 kg), ground to a fine powder, were macerated 15 days with methanol (20 l). The total extract was evaporated to 4 l at 50℃ under reduced pressure, water (4 l) was added and the resulting suspension was kept at 0℃ for 2 weeks, filtered, concentrated to 4 l and refiltered through filter paper. The aqueous solution was then made basic to pH 9.5 with ammonium hydroxide and extracted with chloroform (3×1,000 ml) to give the total alkaloid.

The total alkaloid was dissolvd in a small volume of the upper phase of solvent system (n-butanol: water: acetic acid=4:5:1), and added to a column (2.5 Cm, i.d) fairly tightly slurry-packed in solvent system with silica gel (Merck, through 70 ~230 mesh). Elution proceeded with the upper phase of solvent system (n-butanol: water: acetic acid=4:5:1): fractions were cut on the basis of fluorescence under long wave ultraviolet light. The first fraction contained nonalkaloid material (blue fluorescence). Further elution gave three yellow bands; fraction 2 and 3 (Rf 4.7~3.9) were evaporated under reduced pressure and rechromatographed. The first fraction was concentrated and crystallized from acetone to give white crystal. Mass spectrum was obtained on a Hewlett parkard GC/Mass spectrometer using an electron impact method (Fig. 1).

Duodenum preparation

The longitudinal smooth muscle isolated from the rat duodenum was used in this study. In each experiment, a segment of muscle approximately 3 cm long was suspended in a muscle bath (vol, 5 ml) that contained Tyrode's solution maintained 37± 1℃. The Tyrode's solution had following composition (mM): NaCl, 137; KCl, 2.7; $CaCl_2$, 1.8; $NaHCO_3$, 12 and glucose, 5.6, and bubbled with 100% oxygen. Other bathing solution that were used in this investigation include; ① a calcium-depleted solution in which 1.8 mM $CaCl_2$ was not added to the Tyrode's solution; ② a potassium depolarizing solution in which all the NaCl of the calcium-depleted solution replaced by an equal molar concentration of KCl.

Isometric contraction of the longitudinal muscle was measured by means of a Narco 7173

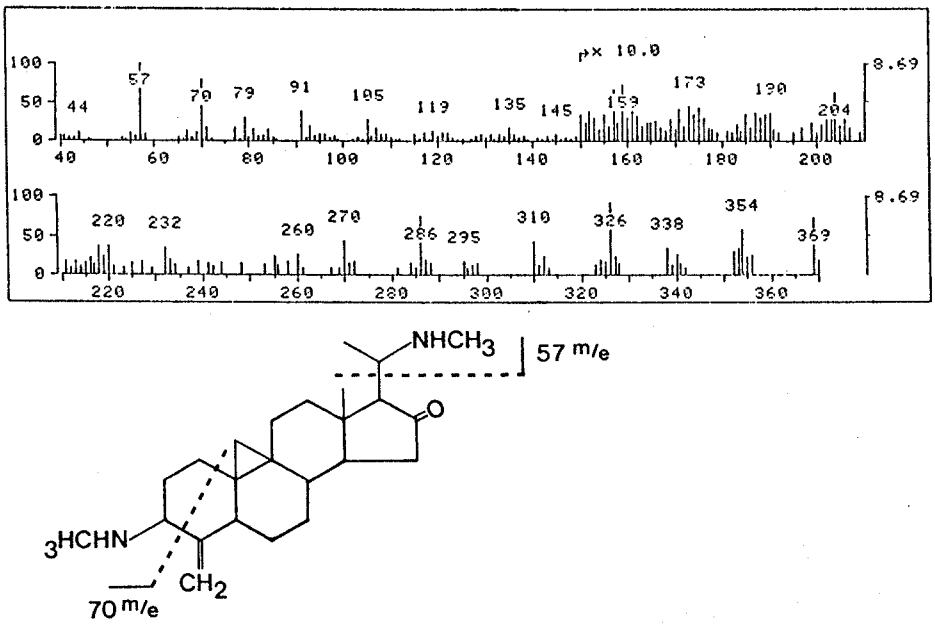

Fig. 1 GC-Mass spectrum and structure of cyclobuxine E.

Myograph transducer and recorded on a Narco physiograph. Initial tension on the muscle was set at 1.0 g. Drugs used were acetylcholine chloride (sigma), EDTA (sigma), verapamil (Jong Kyun Kang LTD. Co) and cyclobuxine E (crystallized in our Lab.). All drugs were dissolved in the bathing solution. Drug concentrations discribed in this paper are expressed as final concentration in organ bath.

RESULTS

A dose-dependent decrease of peak tension in the normal contraction was observed when cyclobuxine E was added to the organ bath.

As the concentration of cyclobuxine E was increased from $3.2\times10^{-5}$to 3.2×10^{-3}M, the peak tension of the Ach-induced contraction were dose-dependently decreased. The dose response curve was shown (Fig. 2).

After the longitudinal muscle of the rat duodenum were immersed in a calcium-depleted Tyrode's solution for 50 min and in a calcium-depleted potassium-depolarizing solution for an additional 10 min, a concentration of 1.8 mM $CaCl_2$ was addd to the muscle bath. The addition of the calcium immediately elicited the rapid, highly transient phasic portion of the tension response (Fig. 3, A). After some degree of relaxation occurred, the muscle underwent a second more gradual rise in tension, referred to as the tonic response (Fig. 3, B). The gradual decrease in magnitude of component B was followed for 100~120 min. The development of these two types of tension change in the presence of a high potassium concentration has been observed previously by numerous investigators (Imai and Takeda, 1967; Syson and Huddart, 1973; Triggle and Triggle, 1976; Gabella, 1978; Hurwitz *er al.,* 1980; Sally *er al.,* 1985). Both components of contractile response were found to be sensitive to cyclobuxine E. In the presence of 1.6×10^{-3}M cyclobuxine E (Fig. 4, a), phasic response and tonic response were inhibited over 70% (n=3). In the presence of 3.2×10^{-3}M cyclobuxine E, both components were completely inhibited. In the presence of 4×10^{-6}M verapamil (Fig. 4, b), they were inhibited over 80 % (n=3). However, 1×10^{-7}M atropine was added to the bathing medium 5 min before the $CaCl_2$ was introduced. The record in figure 4(b) demonstrates that the presence of atropine did not appreciably alter the magnitude of the biphasic contractile response from it control value.

Furthermore, in another set of experiments, 3.2

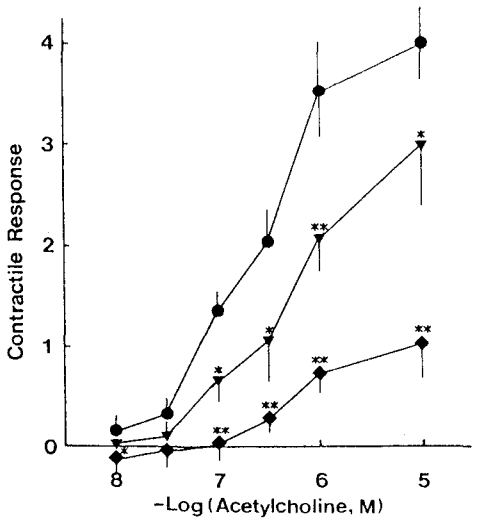

Fig. 2. The effect of cyclobuxine E on the peak tension of the duodenal contraction induced by acetylcholine. The symbols correspond to the following; ●, control; ▼, 3.2×10^{-4}M cyclobuxine E; ■, 3.2×10^{-3}M cyclobuxine E. *p<0.05 and **p<0.01 compare to control.

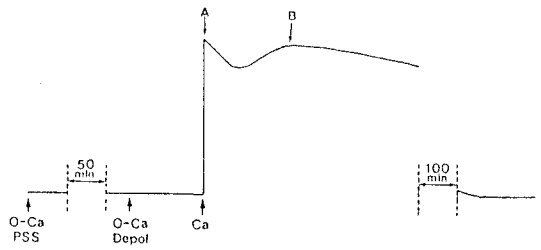

Fig. 3. Isometric contractions of the longitudinal muscle. The label (O-Ca Depol) and arrow refer to the point at which the muscle was immersed in a calcium-depleted, potassium-depolarizing solution. The label (Ca) and arrow refer to the point at which 1.8 mM $CaCl_2$ was added to the bathing medium. The components A and B of the mechanical response that was elicited are labeled at the appropriate places on the recording. See text for further explanation.

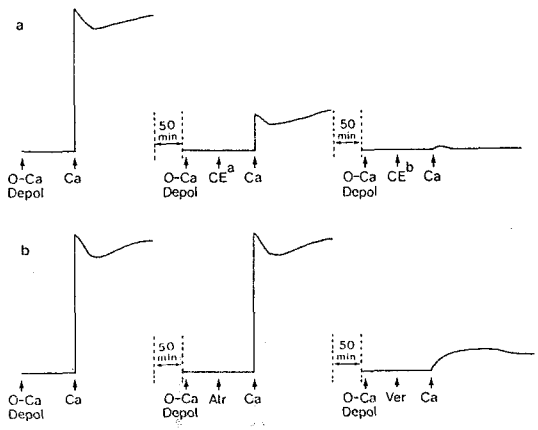

Fig. 4. Isometric contractions of the longitudinal muscle. At a, the left segment of the recording represents a control contraction elicited by 1.8 mM $CaCl_2$ (Ca) after the muscle had been suspended in a calcium-depleted solution for 50 min and in a calcium-depleted, potassium-depolarizing solution (O-Ca Depol) for an additional 10 min. The middle and right segments of the recording show the mechanical response developed by the muscle after it was subjected to the same treatment, but in addition 1.6×10^{-3}M (CE^a) and 3.2×10^{-3} M (CE^b) cyclobuxine E were added to the bathing medium 5 min before the 1.8 mM $CaCl_2$ was introduced. At b, the procedure employed was similar to that described above except that 2.0×10^{-7}M atropine and 4.0×10^{-6} verapamil (Ver) were added instead of cyclobuxine E.

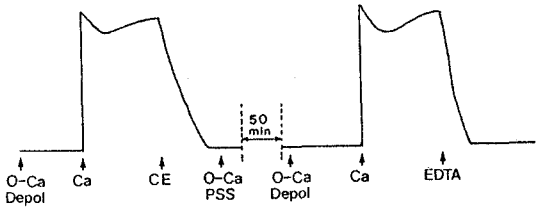

Fig. 5. Isometric contractions of the longitudinal muscle. The left segment of the recording represents a control contraction elicited by 1.8 mM $CaCl_2$ (Ca) after the muscle had been suspended in a calcium-depleted solution for 50 min and in a calcium-depleted, potassium-depolarizing solution (O-Ca Depol) for an additional 10 min. During a control response, 3.2×10^{-3}M cyclobuxine E was added to the muscle bath at the point at which the tonic response had reached its maximum tension. At the right segment, the procedure employed was similar to that described above except that 4.8×10^{-3}M EDTA was added instead of cyclobuxine E. The term (O-Ca PSS) and arrow refer to the point at which the muscle was washed and immersed in a calcium-depleted physiological salt solution for 50 min.

$\times10^{-3}$M cyclobuxine E was added to the bathing medium after component B reached it highest magnitude. Under these conditions, the muscle exhibited a rapid loss of tension (Fig. 5). The addition of 4.8 mM EDTA showed the same effect (Fig. 5).

DISCUSSION

The present findings show that in longitudinal smooth muscle isolated from the rat duodenum, cyclobuxine E relaxed dose-dependently the muscle and inhibited the contractile response to acetylcholine.

There may be as many as four different sources of Ca^{++} for contraction; (a) calcium entering through the AP channel, (b) calcium entering through ROCs, (c) calcium bound to receptors, (d) release of calcium from endoplasmic reticulum, inner surface of cell membrane, and possibly also mitochondria (Bolton, 1979).

The isolated muscle of the rat duodenum when exposed to a high potassium ion concentration, undergo a complex series of mechanical changes. Initially, a rapid, highly transient increase in tension (component A) develops. This is followed by a second, slow increase in tension (component B) which subsequently diminishes in magnitude over a prolonged period of time (100~120 min) (Fig. 3). Numerous investigators studied on the biphasic response induced by a high potassium ion concentration in a number of smooth muscle preparation. It is conceivable that the phasic response may have been initiated by an immediate release of calcium ions from intracellular stores and that the tonic response may have developed as a result of an influx of calcium ions from the extracellular environment. This possivility was negated by some investigators (Golenhofen and Lammel, 1972; Haeusler, 1972; Kohlhardt *et al.*, 1972; Van Breeman *et al.*, 1977).

Golenhofen and co-workers (Boev *et al.*, 1976; Golenhofen, 1976) seem to have uncovered at least two types of calcium channels. One group of channels was labeled the P system; the other the T system. Hurwitz *et al*, (1980) reported that the existance of the two types of calcium channels, which are activated specially by a potassium, did not fall into the P and T categories delineated by Golenhofen and co-workers.

Both components of the contractile response were found to be sensitive to the calcium antagonist, verapamil and not to atropine. In the presence of 4×10^{-6}M verapamil, they were inhibitd over 80 %. Furthermore, as EDTA was added to the bathing medium after component B reached its highest magnitude, the muscle exhibited a rapid loss of tension. These observation, plus the fact that the potassium-induced tension changes were not elicited until calcium ion was added to the bathing medium, provide strong evidence that both components of the mechanical response are dependent upon an influx of calcium ions into the smooth muscle cell from the external medium.

Cyclobuxine E inhibited significantly two components which were elicited by a potassium-induced sustained depolarization of the longitudinal muscle membrane. In addition, cyclobuxine E, introduced at a point when the tonic response had reached its maximum level, caused the muscle to exhibit a rapid loss of tension.

The observations made in this study raise the possibility that the inhibitory action of cyclobuxine E in isolated smooth muscle may be due to blocking calcium channels.

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= 국문초록 =

흰쥐 장관에 있어 칼륨에 의해 활성화되는 칼슘 채널에 대한 Cyclobuxine E의 영향

순천향대학 의학부 약리학교실

이종화 · 권준택 · 조병헌 · 최규홍 · 김유재 · 김종배 · 김천숙 · 차영덕 · 김영석

본 연구실에는 최근 수년동안 말라리아, 성병등에 민간약으로 사용되어 온 회양목(*Buxus microphylla* var. *koreana* Nakai)으로 부터 다수의 물질을 분리하여 그 약리작용을 검색하여왔다. Coumarin의 유도체인 buxuletin은 이뇨 작용이 있음이 인정되었으며, steroid 성 alkaloid인 Cyclobuxine D($C_{25}H_{42}ON_2$)는 항염증작용, 흰쥐에서 심박동수 감소작용 및 적출 평활근 이완 작용을 나타냈다.

본 연구에서는 회양목에서 Cyclobuxine D의 유도체인 Cyclobuxine E(C_{24} H_{38} ON_2)를 분리하여 그 구조를 이화학적인 방법으로 규명하였으며 흰쥐의 십이지장 평활근에서 acetylcholine에 의해 유도되는 수축 작용에 대한 영향과 높은 칼륨 이온에 의해 활성화되는 칼슘 채널에 대한 Cyclobuxine E의 영향을 관찰하였다. Cyclobuxine E는 적출 십이지장 평활근에서 acetylcholine의 수축작용을 현저히 억제하였으며, Calcium-depleted potassium-depolarizing 용액에 담근 후 $CaCl_2$를 가함으로써 나타나는 이중적인 수축작용을 용량적으로 차단하였다. 이상의 십이지장 평활근에 대한 Cyclobuxine E의 작용은 Cyclobuxine E가 칼륨에 의해 활성화되는 칼슘 채널 (아마, voltage-dependent calcium channel)을 통한 칼슘의 세포막 통과를 차단하므로 인해 나타남을 시사한다.