ON THE INHIBITORY MECHANISM OF BASSIANOLIDE, A CYCLODEPSIPEPTIDE, IN ACETYLCHOLINE-INDUCED CONTRACTION IN GUINEA-PIG TAENIA COLI

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Abstract—The effect of bassianolide (BASS), a cyclodepsipeptide, was investigated on mechanical response, membrane potential, intracellular Na and K contents and ^{45}Ca uptake in response to acetylcholine (ACh) in guinea-pig taenia coli. BASS (10^{-5} M) as well as verapamil (5×10^{-7} M) and papaverine (3×10^{-5} M) selectively inhibited the tonic component of the contraction induced by ACh (10^{-5} M), but scarcely affected the phasic one. In contrast, atropine (3×10^{-8} M) inhibited both components of contraction. BASS did not modify the change in membrane potential by ACh. BASS, ACh and the combination of both did not influence the intracellular Na and K contents and the ^{45}Ca uptake. These data show that BASS seems unlikely to have the property of an ionophore. BASS slightly inhibited both the tonic and phasic components of contraction induced by 60 mM K in a non-selective manner, though verapamil and papaverine inhibited the tonic component more potently than the phasic one. Verapamil decreased the increased ^{45}Ca uptake in the muscle soaked in 60 mM K medium, but BASS did not. Since BASS selectively inhibits the tonic component of the ACh-induced contraction, the inhibitory mechanism of BASS seems to be different from that of verapamil, papaverine or atropine; and the mechanism may be beyond the interactions with a binding activity of ACh to the muscarinic receptor, membrane potential and the contractile machinery of the intestinal smooth muscle.

Bassianolide (BASS) is a cyclodepsipeptide isolated from the cultured mycelia of *Beauveria bassiana*, a pathogenic organism to the silk worm (1, 2). Nakajyo et al. (3) have reported that BASS inhibits the isotonic contractions induced by cholinergic drugs, histamine, 5-hydroxytryptamine and prostaglandin E_2 ; but it slightly inhibits the contraction induced by barium (Ba) or high

concentration of potassium (high K) in the ileal longitudinal muscle preparation of guinea-pig. Moreover, BASS inhibited the contractions induced by adrenergic and cholinergic drugs and histamine, though it hardly influenced the contraction by Ba or high K in guinea-pig vas deferens (3). On the other hand, Nakajyo et al. (4) have shown that BASS inhibited the contraction

by high K, Ba or tetraethylammonium as well as adrenergic drugs in guinea-pig aorta. They have suggested that BASS inhibits the contractile actions of drugs which have an effect upon a selective site or receptor of the smooth muscle cell in the ileum and the vas deferens, except for guinea-pig aorta.

Although BASS inhibited the contraction induced by ACh in the ileal longitudinal muscle or vas deferens of guinea-pig, BASS did not affect a twitch stimulated by electric shock in a mouse phrenic nerve-diaphragm muscle preparation and in a frog sciatic nerve-sartorius muscle preparation. BASS also had no effect on the ACh-induced contracture in a frog rectus abdominis preparation (5). They have suggested that BASS depresses the muscarinic response to ACh, but not the nicotinic (N_2) one. Furthermore, BASS did not affect the binding activity of [$3\text{-}^3\text{H}$] quinuclidinil benzylate (^3H -QNB) to the muscarinic receptor of the crude synaptosomal membrane preparation of the cerebral cortex of guinea-pig or mouse. Accordingly, the inhibitory action of BASS on the muscarinic response in smooth muscle, unlike atropine, may not be due to an inhibition of ACh binding to the receptor (5).

In the present study, we investigated the effect of BASS on changes by ACh in the mechanical response, membrane potential, intracellular Na or K contents and ^{45}Ca uptake in guinea-pig taenia coli. Moreover, the experiment was performed to compare the effect of BASS with that of verapamil, papaverine or atropine, which is a typical relaxant in intestinal smooth muscles. The results showed that BASS selectively inhibited the ACh-induced tonic contraction, though it slightly inhibited both the tonic and phasic components of the 60 mM K-induced contraction.

Materials and Methods

Measurement of mechanical and electrical

responses: Male guinea-pigs weighing 400–500 g were killed by a blow on the head and bled to death. Smooth muscle strips of taenia coli, approximately 15 mm long in situ, were removed. The muscle preparation was suspended in a modified Tyrode solution exposed to a gas mixture of 95% O_2 and 5% CO_2 . The composition of the solution (mM) was NaCl, 136.8; KCl, 5.4; CaCl_2 , 2.5; MgCl_2 , 1.0; NaHCO_3 , 11.9 and glucose, 5.5. The organ bath was kept at $36 \pm 1^\circ\text{C}$ and pH 7.2. The muscle strip was attached to the arm of a force displacement transducer (Nihon Kohden). After the equilibration of the muscle in the normal solution for 30 min, the resting tension was adjusted to 0.5 g. The contractile tension of the muscle strip was recorded isometrically. Then an appropriate volume of 1 M KCl solution was added hypertonically to the organ bath to give a final concentration of 40 or 60 mM K. The maximal size of the muscle contraction induced by an agonist was regarded as 100%. Since the effect of a 15 min exposure to BASS proved to be optimal, antagonists or BASS were applied 15 min before the application of agonists, unless otherwise stated.

The membrane potential was measured by extracellular recording using the single sucrose gap method as described by Bülbring and Burnstock (6).

Determination of Na and K contents: For estimates of cellular Na and K contents, the "La method", developed by van Breemen (7), was employed. To be consistent with the contraction experiments, tissues were pre-treated with antagonist for 15 min before exposing them to either ACh (10^{-5} M) or high K solution also containing antagonist. After incubation of the muscle strips in various test solutions, the strips were then transferred to a La-sucrose solution containing isotonic sucrose and 1 mM LaCl_3 , at 0.5°C . After the La treatment for 30 min, each of the strips was blotted between filter

papers and weighed. It was then ashed in a quartz tube with 0.5 ml of a mixture of equal amounts of HNO_3 (61%) and HClO_4 (60%), overnight at 180°C . The samples were then redissolved in 0.01 N HCl solution. Na and K contents in the samples were measured by flame photometry and expressed as mmol/kg wet wt. of this tissue. Since the La wash removed a Na fraction bound to the cell membrane surface, the value of cellular Na content in this paper was lower than a value obtained from the total tissue Na content subtracting Na content in the extracellular space.

Experiment of ^{45}Ca uptake: ^{45}Ca uptake into the smooth muscle cells was measured by a modified "La method" which was developed by Karaki and Weiss (8). In an experiment to study the rate of Ca uptake, the tissues which had been incubated in normal medium containing 2.5 mM Ca for 2 hr, were exposed to solutions containing ^{45}Ca for short periods of time, 3 or 6 min. The amount of ^{45}Ca entering the tissue during a short period can be assumed to be primarily due to Ca influx (9). The time schedule of incubation in the various test solutions was almost the same as that used in the experiment for "Determination of Na and K contents". After incubation with various test solutions labelled with ^{45}Ca (5 $\mu\text{Ci}/\text{ml}$, New England Nuclear), the muscle strips were exposed to the La solution (LaCl_3 , 73.8 mM; glucose, 5.5 mM and Tris-HCl, 11.9 mM) at 0.5°C for 60 min. The strips were then placed in scintillation vials containing 0.5 ml of tissue solubilizer (Soluene-350, Packard) and were digested by warming overnight at 55°C . The sample was then mixed with 5 ml of dioxane based scintillator, and radioactivity was determined using a liquid scintillation spectrometer (Tri-Carb 3380, Packard). Intracellular ^{45}Ca content was expressed as $\mu\text{mol}/\text{kg}$ tissue.

Drugs used: BASS isolated from the

cultured fungi *Beauveria bassiana* is a cyclodepsipeptide composed of four molecules of 1-N-methylleucine and d- α -hydroxy-isovaleric acid. The molecular formula is $(\text{C}_{12}\text{H}_{21}\text{NO}_3)_4$ and its molecular weight is 908. It is a white crystalline substance which is insoluble in water and soluble in organic solvents. BASS was dissolved in ethanol at the desired concentration, and the ethanol solution was applied in a volume of less than 0.02 ml in the 15 ml organ bath solution. The same volume of ethanol was applied as a control to confirm that ethanol itself had no influence upon the preparation in any of the types of experiments. The following drugs were used: acetylcholine chloride (ACh, Daiichi Seiyaku), atropine sulphate (ATR, Wako Junyaku), verapamil (Eisai) and papaverine hydrochloride (Daiichi Seiyaku).

The results are presented as the mean values $\pm$ S.E.M. for n experiments. Differences were analyzed using the Student's t -test to determine significant differences.

Results

Effect of BASS on ACh-induced contraction: An application of 10^{-6} M ACh induced a phasic contraction (6 g) followed by a tonic one (4 g) in spontaneously active smooth muscles of guinea-pig taenia coli. ACh at higher concentrations (10^{-5} – 10^{-4} M) enhanced both the contractile responses. The typical responses of the six muscle strips to ACh (10^{-5} M) are shown in Fig. 1A & B. A pretreatment with 5×10^{-7} M verapamil for 15 min depressed the spontaneous contractions in amplitude and frequency and inhibited 25–30% of the phasic response to ACh ranging from 10^{-6} to 10^{-4} M, while it inhibited almost completely the tonic response to 10^{-6} M and 10^{-5} M ACh, but 7% of the tonic response remained at 10^{-5} M ACh (Figs. 1A & 2). Papaverine (3×10^{-5} M) exhibited similar results to verapamil as

Fig. 1. Effect of verapamil (5×10^{-7} M) (A) and bassianolide (BASS, 10^{-5} M) (B) on a contraction induced by acetylcholine (10^{-5} M) or 60 mM K in guinea-pig taenia coli. All drugs were pretreated 15 min before the application of acetylcholine or 60 mM K in each muscle strip.

shown in Fig. 2. When the muscle was pretreated with 3×10^{-8} M atropine, the antagonist slightly affected the spontaneous activity, but completely blocked both the tonic and phasic components of contraction induced by 10^{-6} M ACh. However, increasing ACh concentration gradedly developed tensions of both the components in the presence of atropine which shows a competitive antagonism (Fig. 2). On the other hand, the phasic response to 10^{-6} to 10^{-4} M ACh was not significantly depressed by a

pretreatment with 10^{-6} M BASS for 15 min, but the tonic responses to ACh at the three concentrations were inhibited to 80%. These data are not shown in the figure. Further, 10^{-5} M BASS inhibited the spontaneous activity and 28%, 17% or 16% of the phasic response to 10^{-6} M, 10^{-5} M or 10^{-4} M ACh, respectively. BASS (10^{-5} M) also inhibited 96%, 88% or 76% of the tonic response by 10^{-6} M, 10^{-5} M or 10^{-4} M ACh, respectively (Figs. 1B & 2). An exposure to 10^{-6} M BASS for 60 min or to 10^{-5} M BASS for 30 min did not increase the inhibitory effects on the ACh induced contraction.

In another series of experiments, a transient contraction by an application of 60 mM K showed approximately 8 g of tension followed by a tonic one of almost the same size. The phasic component was inhibited to 50% by the pretreatment with 5×10^{-7} M verapamil for 15 min, and the tonic one was completely inhibited (Figs. 1A & 2). Since papaverine at a concentration of 3×10^{-5} M inhibited the phasic component of the high K-induced contraction to 44% and the tonic one to 9%, papaverine as well as verapamil may selectively inhibit the tonic contraction of the muscle in high K medium. Although atropine slightly depressed the phasic contraction, it did not affect the tonic one (Fig. 2). Atropine which selectively inhibits the ACh binding to the muscarinic receptor did not inhibit both the phasic and tonic components of the high K-induced contraction (Fig. 2). After the pretreatment with BASS at a concentration of 10^{-6} M or 10^{-5} M for 15 or 30 min, BASS inhibited 20% of both the phasic and tonic components in the high K-induced contraction; BASS inhibited somewhat both the phasic and tonic components to the same extent, in a different manner from verapamil and papaverine (Figs. 1B & 2). Although verapamil and papaverine were shown to be equiactive in the inhibitions of the tonic components of ACh and 60 mM K responses,

Fig. 2. Effect of verapamil, papaverine, atropine or bassianolide (BASS) on the phasic (opened column) and tonic (dotted column) components of the contraction induced by acetylcholine (10^{-6} – 10^{-5} M) or 60 mM K in guinea-pig taenia coli. Ordinate: relative contraction (%), the maximal size of the phasic or tonic components of a contraction induced by agonist was regarded as 100%. Six muscle strips were employed in each experiment.

BASS displayed a selectivity of inhibition towards the ACh-induced tonic contraction.

Effect of BASS on membrane potential:

The membrane potential of the smooth muscle was measured at 32°C by the single sucrose-gap method. The muscle spontaneously depolarized the membrane accompanying spike discharges as shown in Fig. 3. An

application of 10^{-5} M BASS or 10^{-7} M atropine induced a slight hyperpolarization and a disappearance of spike discharges. ACh (10^{-5} M) caused a depolarization of approximately 14 mV and increased the frequency of spike discharges. An application of high K caused depolarization of 12 mV and produced a change in membrane potential

Fig. 3. Effect of atropine (ATR, 10^{-7} M) and bassianolide (BASS, 10^{-5} M) on changes in membrane potential by acetylcholine (ACh, 10^{-5} M) or 40 mM K in guinea-pig taenia coli. In A, B and C, different strips were employed. In the sucrose-gap apparatus, the flow rate was 6 ml/min. The distance is approximately 30 cm between the muscle bath and the cock which exchanges the test solution.

similar to that by ACh. Pretreatment with 10^{-7} M atropine decreased the changes by ACh, but did not influence those by high K. On the other hand, pretreatment with BASS (10^{-5} M) did not modify the changes in membrane potential by either ACh or high K (Fig. 3).

Effect of BASS on intracellular Na and K contents: In the smooth muscle of taenia coli, intracellular Na and K were 5.0 ± 0.4 mmol/kg wet wt. and 61.1 ± 1.0 mmol/kg wet wt., respectively. ACh or BASS was applied for 30 min to muscle soaked in the medium in the presence or absence of ouabain at a concentration of 2×10^{-5} M. ACh (10^{-5} M) did not change the values of intracellular Na and K contents (Table 1). BASS (10^{-5} M) with or without ACh (10^{-5} M) also did not modify them. Ouabain (2×10^{-5} M) increased intracellular Na content to 22.7 ± 1.9 mmol/kg wet wt. and reduced intracellular K content to 39.3 ± 3.1 mmol/kg wet wt. ACh (10^{-5} M), BASS (10^{-5} M) or the combination of both

did not influence the ion contents of the muscle treated with ouabain.

Effect of BASS on ^{45}Ca uptake: Amount of ^{45}Ca uptake into the smooth muscle of taenia coli was 27.7 ± 2.2 $\mu\text{mol/kg}$ wet wt. of tissue for 3 min or 58.6 ± 6.1 $\mu\text{mol/kg}$ wet wt. for 6 min in the normal medium. Sixty mM K increased ^{45}Ca uptake to twice that of the control (Table 2). Verapamil at 10^{-6} M decreased the increased ^{45}Ca uptake back to the control level; however, BASS (10^{-5} M) did not influence it. On the other hand, 10^{-5} M ACh slightly increased ^{45}Ca uptake of the muscle for 3 min, but did not for 6 min. The result is consistent with the data reported by Triggie and Triggie (10) that cis-2-methyl-4-dimethylaminomethyl-1,3-dioxanmethiodide (CD), a typical muscarinic agent at a concentration of 5×10^{-7} M, increased ^{45}Ca uptake in ileal longitudinal smooth muscle during the phasic component of CD response, but not during the tonic one. The ^{45}Ca uptake into the muscle treated with ACh (10^{-5} M) was

Table 1. Changes in cellular Na and K contents of guinea-pig taenia coli by acetylcholine, bassianolide and the combination of both, in presence or absence of ouabain

Treatment	Cellular ion contents (mmol/kg wet wt.)	
	[Na] _i	[K] _i
Normal	5.0±0.4	61.1±1.0
ACh (10 ⁻⁵ M)	4.5±1.3	60.8±2.5
BASS (10 ⁻⁵ M)	5.2±1.3	58.1±3.7
ACh (10 ⁻⁵ M)+BASS (10 ⁻⁵ M)	4.2±0.8	62.6±4.1
Ouabain (2×10 ⁻⁵ M)	22.7±1.9	39.3±3.1
ACh (10 ⁻⁵ M)+Oua (2×10 ⁻⁵ M)	23.9±1.2	38.7±3.7
BASS (10 ⁻⁵ M)+Oua (2×10 ⁻⁵ M)	19.7±2.4	40.9±3.6
ACh (10 ⁻⁵ M)+BASS (10 ⁻⁵ M) + Oua (2×10 ⁻⁵ M)	25.2±0.4	35.9±1.5

The muscles were incubated in normal medium for 1 hr, then they were soaked for 30 min in the medium containing 10⁻⁵ M ACh with or without ouabain (Oua, 2×10⁻⁵ M). Bassianolide (BASS, 10⁻⁵ M) was added 12 min before the ACh treatment. Ouabain significantly increased [Na]_i and decreased [K]_i (P<0.05); however, a significant difference was not found between them in the experiment. Each value is the mean of six results±S.E.M.

Table 2. Effect of bassianolide, verapamil or atropine on ⁴⁵Ca uptake into the smooth muscle of guinea-pig taenia coli treated with 60 mM K or acetylcholine

Treatment	⁴⁵ Ca uptake (μmol/kg wet wt.)	
	3 min	6 min
Normal	27.7±2.2	58.6±6.1
60 K	52.7±4.1*	96.4±13.0*
60 K+Ver (10 ⁻⁶ M)	21.8±1.7**	51.1±6.4**
60 K+BASS (10 ⁻⁵ M)	48.6±3.0	94.7±6.5
ACh (10 ⁻⁵ M)	33.8±1.6*	58.5±3.2
ACh+Ver (10 ⁻⁶ M)	24.0±1.8**	39.9±3.1**
ACh+Atr (10 ⁻⁶ M)	28.6±1.9	58.3±7.0
ACh+BASS (10 ⁻⁵ M)	33.4±1.4	60.2±5.0

The muscles were incubated in normal medium for 2 hr. ACh (10⁻⁵ M) or 60 mM K was simultaneously applied to the muscle with ⁴⁵CaCl₂. Bassianolide (BASS, 10⁻⁵ M), Verapamil (Ver, 10⁻⁶ M) or atropine (Atr, 10⁻⁶ M) was added 12 min before ⁴⁵Ca loading and kept during the loading. Each value is the mean of six results±S.E.M. *: Significant difference from the value in normal sol. (P<0.05).

** : Significant difference from the value in a solution containing ACh (10⁻⁵ M) or 60 mM K (P<0.05).

decreased by verapamil (10⁻⁶ M), but it was not decreased by atropine (10⁻⁶ M) or BASS (10⁻⁵ M). The decrease of ⁴⁵Ca uptake by verapamil below the control level may be caused by blocking the basal Ca influx of the muscle and/or by an increase of Ca efflux by ACh which is not modified by verapamil. Since the cholinergic effect on

Ca efflux of the smooth muscle has not been established, the latter assumption is ambiguous. Since an increase in ⁴⁵Ca uptake by ACh for 6 min was not measured by this method, we could not obtain evidence about whether BASS modifies the Ca uptake during the ACh-induced tonic contraction or not.

Discussion

In the previous paper (5), it was reported that BASS inhibited the muscarinic response to ACh but not the nicotinic (N_2) one and that BASS, unlike atropine, did not affect the binding of ACh to the muscarinic receptor of the crude synaptosomal preparation of the brain. In the present paper, atropine which inhibits the binding activity of ACh to the muscarinic receptor inhibited both the phasic and tonic contractions induced by ACh in guinea-pig taenia coli. However, BASS, verapamil and papaverine almost completely inhibited the tonic component, but only partly inhibited the phasic one. Moreover, ACh bound to the receptor accelerated action potential discharge and depolarized the membrane with higher concentrations in the smooth muscle of taenia coli (6, 11). Atropine inhibited these changes in the membrane potential, but BASS did not. From these data, it seems likely that the inhibition of the ACh-induced contraction by BASS is not due to the changes in binding activity of ACh to the muscarinic receptor or in electrical properties of the cell membrane of smooth muscle.

There are many reports concerning the effect of muscarinic agents on the movement of monovalent cations. Bolton (12) has reviewed that the muscarinic agents increased ^{22}Na influx into and ^{24}K efflux from intestinal smooth muscle. However, these agents did not influence the Na level, but they reduced the K level of the muscle. In the present data, ACh, BASS and the combination of both did not change the intracellular Na and K contents of taenia coli in the presence or absence of ouabain. Although these results were not consistent with the review, the BASS inhibition of the contraction may not be related to the change in the ion contents or movement during the ACh response.

As Bolton (12) has reviewed, data on the

effect of muscarinic agents on Ca fluxes have proved to be ambiguous: Activation of the muscarinic receptor has reported to increase Ca influx, although other workers have not found this. Some workers have found Ca efflux to be increased, and others found it unchanged by the muscarinic agent in polarized smooth muscle. However, a current concept has been accepted that the Ca involved in the tonic component of ACh-induced contraction is derived from an extracellular source, while Ca supporting the phasic component may be derived from a releasable intracellular source. In this study, ^{45}Ca uptake at the 6th min which corresponds to the tonic response was increased by 60 mM K, but it was not affected by ACh (10^{-5} M). The lack of extra ^{45}Ca uptake during the tonic component of ACh contraction appears inconsistent with the high dependence of this component upon extracellular Ca. This discrepancy may be explained by a concept that the ^{45}Ca influx specially associated with the mechanical response might not be readily measured by this tracer technique. Accordingly, the present data could not provide substantial evidence indicating an inhibition by BASS or verapamil on the ACh action on Ca metabolism. However, the fact that BASS did not modify the changes in membrane potential of the muscle by ACh suggests that BASS may not interfere with the electrogenic Ca influx increased by ACh.

On the other hand, an elevation of external K in medium depolarized the smooth muscle, with an accompanying increase in amplitude and frequency of spike discharge, then induced a contraction. The K-induced contraction also consists of phasic and tonic components. Increase of Ca uptake during high K contraction of the guinea-pig intestine has been reported by many investigators (10, 13–17), and they have suggested that extracellular Ca is utilized during the tonic

component of the contraction and that the phasic contraction is mainly due to a Ca release from an intracellular store. However, an application of high K appears to release a significant amount of Ca from a storage site which is different from that derived by a muscarinic agent (18, 19). In the present data, verapamil inhibited the tonic contraction and the increased ^{45}Ca uptake in high K medium. However, BASS slightly inhibited the tension development both in the phasic and tonic phase in a non-selective manner, and BASS did not affect the electrical properties and the increase in the ^{45}Ca uptake into the muscle in high K medium, which seems to be different from verapamil.

In the present data, papaverine as well as verapamil selectively inhibited the tonic component of ACh- or 60 mM K-induced contraction. This effect of papaverine was consistent with that reported in other papers (20, 21). Since BASS did not remarkably inhibit the 60 mM K-induced tonic contraction, the action of BASS seems to be also different from that of papaverine which inhibits phosphodiesterase activity (22–24) or mitochondrial respiration (25, 26).

Suzuki and his co-workers (1, 2) have reported that BASS is one of the cyclodepsipeptides which has a chemical structure with oxygen atoms on its ring. Such cyclodepsipeptides generally have the properties of an ionophore (27). In the experiments of membrane potential, intracellular Na and K contents and ^{45}Ca uptake, BASS did not change the permeability of monovalent or divalent cations through the cell membrane. It is unlikely that BASS has any ionophoric properties towards the cell membrane of smooth muscle.

In summary, BASS selectively inhibited the tonic component of ACh-induced contraction, but slightly inhibited both the phasic and tonic contractions by 60 mM K in a non-selective manner. The mechanism

through which BASS inhibits the tonic response to ACh seems to be different from that of verapamil or papaverine which inhibited both the tonic responses to ACh and 60 mM K, and the mechanism seems not to involve changes in the binding activity of ACh to the muscarinic receptor, in membrane potential by ACh and in the contractile machinery of the intestinal smooth muscle.

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