Effects of tetrodotoxin on relaxation of pig duodenal circular muscle induced by C-terminal fragments of cholecystokinin

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- 1 The mechanisms by which cholecystokinin (CCK)-C-terminal peptides relax the isolated duodenal circular muscles of pigs were compared with mechanisms involving high potassium and electrical stimulation.
- 2 The relaxation (30%) induced by CCK-8 0.263 nM was completely and non-competitively blocked by tetrodotoxin (TTX) 0.313 μ M, and was enhanced 2–2.5 times by meprobamate 0.158 μ M, tetraethylammonium (TEA) 0.597 mM and ouabain 0.136 μ M.
- 3 The TTX sensitivity was greater for CCK-8 and CCK-6, than for CCK-5 and CCK-4.
- 4 High potassium (11.8–23.6 mM)-induced, or electrically stimulated (0.3–30 Hz) relaxation showed similar characteristics except that they were not antagonized by the CCK antagonist P3-I (168 μ M).
- 5 Apamin (0.385–3.85 μ M) enhanced relaxation induced by 10.6 mM K^+ , but did not affect relaxation induced by CCK-8.
- 6 These results indicate that: (1) CCK-8, K^+ , and transmural electrical stimulation relax duodenal circular muscles by releasing an endogenous inhibitory substance; (2) the latter two do not mediate the release of CCK; and (3) the mechanisms for relaxation by CCK-4 are different from those of CCK-8.

Introduction

Cholecystokinin (CCK) relaxes the lower oesophageal sphincters (Sturdevant & Kun, 1974), the sphincter of Oddi (Sandblom *et al.*, 1935), and the duodenal circular muscles of pigs (Kimura *et al.*, 1983). Two distinct receptors for CCK have been reported: an inhibitory one present in non-cholinergic and non-adrenergic neurones, and an excitatory one in circular muscles (Behar & Biancani, 1977; 1980). The electrophysiological basis of the relaxation is clearly not suppression of Ca^{2+} spikes but hyperpolarization (Kimura *et al.*, 1983).

On the other hand, we demonstrated that duodenal circular muscles were relaxed by high K^+ (Kimura *et al.*, 1984). The relaxation was correlated with hyperpolarization of the muscle membranes and during the K^+ -induced relaxation, electrically evoked Ca^{2+} spikes were not suppressed, nor were the Ca^{2+} spikes produced during the CCK-4-induced relaxation. Tetrodotoxin blocked both high K^+ -induced relaxation as well as hyperpolarization.

In the present study, we have investigated the mechanisms of relaxation produced by CCK, high

K^+ and electrical stimulation-induced relaxation. We have defined further the differences in relaxation mechanism between CCK-8 and CCK-4.

Method

Pigs of either sex weighing about 90–110 kg were stunned by electrical shock and bled in the slaughter house.

The duodenal segments located about 3–5 cm from the bile duct ampulla were isolated. After the mucous membranes surrounding the segments were cut away, the circular muscles were separated from the longitudinal muscles and from serous membranes, and were then dissected into 2×10 mm strips. The circular strips thus obtained were suspended under 0.5 g loading in a 5 ml organ bath filled with modified Krebs solution (mM: NaCl 122, KCl 5.9, $CaCl_2$ 2.5, $MgCl_2$ 1.2, $NaHCO_3$ 15.5 and glucose 11.5). The bath solution was maintained at 37°C, and bubbled with 95% O_2 and 5% CO_2 .

For transmural stimulation, circular muscles were placed through the loops of a pair of electrodes (platinum wire) 5.5 mm apart. Every 20 s rectangular

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pulses (5 ms duration) were delivered at 0.3, 3 and 30 Hz. High K^+ solutions (10.6–47.2 mM) were prepared by replacing NaCl with equivalent amounts of KCl. Low Na^+ solutions (107–27.7 mM) were prepared by replacing NaCl with osmotically equivalent amounts of sucrose. For 'Ca²⁺-free solution' CaCl₂ was omitted from and EGTA (2 mM) added to the bathing solution. Isotonic tension was recorded on a polygraph (W1-180, Nihon Kohden) with an isotonic transducer (TD-112S, Nihon Kohden) coupled to a biophysical amplifier (RB-2, Nihon Kohden). Tension changes from the normal tone were expressed as a percentage of the value obtained with the Ca²⁺-free solution.

The drugs used were tetrodotoxin (TTX, Sankyo), mesaconitine (a gift from Prof. H. Hikino, Dept. of Pharmacognosy, Tohoku University), tetraethylammonium (TEA, Nakarai), apamin (Sigma), ouabain (Merck), CCK-8 (Peptide Institute), CCK-6 and CCK-5 (gifts from Dr G. Tsukamoto, Kanebo Chemical Institute), AOC(tert-amyloxycarbonyl)-CCK-4 (a gift from Dr Y. Ishii, Nihon Kayaku), proglumide-peptide CCK antagonists, P3-I (L-isomer) and P2-II (D) (D-4-carbobenzoxycarbonyl-N,N-dipropylglutamic acid, Phe-NH₂) Kanebo Chemical Institute (Kimura *et al.*, 1987), vasoactive intestinal peptide (VIP, Peninsula Laboratories, Inc.), papaverine HCl (Nakarai), and (±)-isoprenaline HCl (Kaken). TTX, TEA, apamin, ouabain, papaverine, (±)-isoprenaline, and VIP were dissolved in distilled water. Mesaconitine was dissolved in 0.1 N HCl solution, and diluted with distilled water. CCK-8 and P3-I (L-4-benzamido-N,N-dipropylglutamic acid, Asp-Phe-NH₂) were dissolved in 0.1 N Na₂CO₃ solution, and diluted with distilled water. P2-II (D) was dissolved in DMF, and diluted with distilled water. A 0.1 ml volume of the 50 fold concentrated solution was added to the 5 ml bath solution.

Results

Effects of tetrodotoxin on relaxations induced by cholecystokinin C-terminal peptides

The effects of TTX on the relaxation induced by CCK-C-terminal peptides were compared with those produced by high K^+ or electrical (transmural) stimulation. All three kinds of relaxation are accompanied by hyperpolarization (Kimura *et al.*, 1983; 1984), suggesting the involvement of some inhibitory transmitters. The effect of CCK-8 was blocked non-competitively by 0.313 μ M TTX, and completely abolished by 3.13 μ M TTX as shown in Figure 1a. Typical data are shown in Figure 4a. TTX alone in the concentrations shown, had no direct effect on resting tension. The sensitivity to blockade by TTX

Figure 1 (a and b) Log cumulative concentration-relaxation curves to CCK-8 (○, ●) and CCK-6 (▽, ▼) (a), and CCK-5 (△, ▲) and CCK-4 (□, ■) (b) in the presence (closed symbols) or absence (open symbols) of 0.313–31.3 μ M tetrodotoxin (TTX). The relaxation is expressed as a percentage of the maximal reduction in tone on changeover to Ca²⁺-free Krebs solution containing 2 mM EGTA. Each point represents the mean ($n = 3-4$) and vertical lines show s.e.mean.

of the response to CCK-6 was similar to that for CCK-8. In contrast, responses to CCK-5 and CCK-4 were affected to a smaller extent, even by high concentrations of TTX (Figure 1b). Thus, the effectiveness of TTX in suppressing the relaxant effect of various CCK-C-terminal peptides is as follows: CCK-8 $\geq$ CCK-6 > CCK-5 > CCK-4.

Effects of tetrodotoxin on relaxation to high K^+

The effect of TTX on the responses to high K^+ concentrations was investigated as shown in Figure 2a. Na^+ in the nutrient solution was replaced with K^+ to give 11.8, 13.0, 14.2, 17.7, 23.6, 47.2 and 143.4 mM K^+ solutions. The onset of relaxation was 20–30 s after application of a high K^+ solution. The maximal relaxation, which occurred in 17.7 mM K^+ , was estimated to be 82% of that obtained upon changeover to Ca²⁺-free solution containing 2 mM EGTA. K^+ concentrations higher than 23.6 mM caused a weak relaxation followed by a transient contraction. The contraction was measured from the level of tone in Ca²⁺-free solution. The low concentration of TTX (0.313 μ M) completely suppressed the high K^+ -induced relaxation (Figure 2a); a typical effect is shown in Figure 4b.

Figure 2 (a) Log concentration-response curves for the relaxation (○) and contraction (Δ, ▲) induced by increasing concentration of K⁺ in the presence (closed symbols) or absence (open symbols) of 0.313 μM tetrodotoxin (TTX). The relaxations are as in Figure 1. The contractions after relaxation were measured from the level of minimum tone due to deprivation of calcium. Each point represents the mean ($n = 4-11$) and the vertical lines show s.e.mean. (b) Log Na⁺ concentration-response curves for relaxations (○) and contractions (Δ). NaCl was replaced with equivalent amounts of KCl (solid lines) and with sucrose (broken lines). The relaxations and contractions are represented as in (a). Each point represents the mean ($n = 4-11$), and the vertical lines show s.e.mean.

The patterns of responses to high K⁺ were subtly different from those to low Na⁺ without changing the K⁺ concentration (sucrose substitution) as shown in Figure 2b. The onset of relaxation after applying low Na⁺ was comparatively slow (60–90 s); the maximal relaxation was estimated to be 75%. Relaxations to low Na⁺ were observed over a comparatively wide range (76.5–107 mM) of concentrations. These results indicate that the effects of high K⁺ were not mainly due to the decrease in Na⁺ concentration.

Effect of tetrodotoxin on relaxation to transmural stimulation

Circular muscles were stimulated transmurally with trains of pulses (3–3.5 V, 5 ms) with increasing frequency (0.3, 3, and 30 Hz every 20 s). The relaxation response at all frequencies was reduced in a concentration-dependent manner by TTX (0.313 and 3.13 μM) as shown in Figure 3. The higher concentrations of TTX almost completely blocked the relaxation. The sensitivity to TTX of the relaxation in response to field stimulation is thus similar to that observed for CCK-8- or high K⁺-induced relaxation.

Comparison of effects of mesaconitine, tetraethylammonium, apamin, ouabain, and CCK antagonists on CCK-8- and high K⁺-induced relaxation

Mesaconitine, a Na⁺-channel activator (Catterall *et al.*, 1981), TEA, apamin and ouabain were used to investigate the possibility that activation of ion channels and the Na/K pump are involved in CCK-8(0.263 nM)- and high K⁺-induced relaxation. Mesaconitine 0.158 μM, TEA 0.597 mM and ouabain

Figure 3 Log stimulation frequency-relaxation curves in the presence (●) or absence (○) of 0.313 or 3.13 μM tetrodotoxin (TTX) (15 min pretreatment). The circular muscle was stimulated with trains of pulses (3–3.5 V, 5 ms) with increasing frequency (0.3, 3 and 30 Hz every 20 s). The extent of relaxation is represented as in Figure 1. Each point represents the mean ($n = 3$) and the vertical lines show s.e.mean.

Figure 4 (a) Typical records of the responses induced by 0.263 nM CCK-8 (given at ●) with (right hand column) and without (left hand column) 0.313 μM tetrodotoxin (TTX), 0.158 μM mesaconitine, 0.597 mM tetraethylammonium (TEA), and 0.136 μM ouabain. Similar results were obtained consistently in 4–5 separate experiments. (b) Typical records showing the responses induced by 10.6–23.6 mM K⁺ (given at ●) with (right hand column) and without (left hand column) 0.313 μM TTX, 0.158 μM mesaconitine, 0.597 mM TEA, 0.385 μM apamin, and 0.136 μM ouabain. These effects occurred consistently in 2–4 separate experiments.

0.136 μM markedly enhanced the relaxation produced by CCK-8 0.263 nM (Figure 4a), while apamin, even at a high concentration (3.85 μM), had no effect.

The weak relaxation induced by high K⁺, up to 13.0 mM, was greatly enhanced by the above treatments with mesaconitine, TEA, or ouabain but also by apamin (0.385 μM) (Figure 4b). The relaxation due to high K⁺ (up to 23.6 mM) was hardly affected by P3-I (168 μM), a competitive CCK antagonist (Kimura *et al.*, 1987).

The response to transmural electrical field stimulation was enhanced by TEA to a small but significant extent and by mesaconitine (data not shown), but was little affected by ouabain, P3-I or P2-II (D) (19.6 μM).

Papaverine (0.266 μM–0.266 mM) and (±)-isoprenaline (4.04 nM–4.04 μM) relaxed the muscles in a concentration-dependent manner (data not shown); the relaxations induced by 2.66 μM papaverine or by 40.4 nM isoprenaline were unaffected by the above concentrations of TTX, mesaconitine, or TEA (data not shown).

The vasoactive intestinal peptide concentration-relaxation curve compared with that of CCK-8

The concentration-response curve to CCK-8 was compared with that for VIP, a putative candidate as an inhibitory neurotransmitter released by transmural electrical stimulation (Biancani *et al.*, 1984). The potency of VIP was approximately 2 orders of magnitude lower than that for CCK-8 (Figure 5) and the relative intrinsic activity of VIP was 0.93 which is greater than that of CCK-8 (0.78). The VIP-induced relaxation was not suppressed by 3.13 μM tetrodotoxin (data not shown).

Discussion

CCK receptors have been classified as being of 'peripheral' or 'central' type. Peripheral CCK receptors are further classified as neurogenic (duodenum,

Figure 5 Log concentration-relaxation curve of cumulatively administered (every 5 min) vasoactive intestinal peptide (VIP). The relaxation is represented as in Figure 1a. Each point represents the mean ($n = 4$) and the vertical lines indicate s.e.mean. The curve for CCK-8 is replotted from Figure 1.

sphincter of Oddi, and gallbladder), exocrine (amylase secretion), and endocrine (insulin release). CCK-8 has a higher affinity than CCK-4 for the peripheral receptor. Behar & Biancani (1977, 1980) reported that CCK relaxes the lower oesophageal and choledochal sphincter of the cat by releasing an inhibitory transmitter, since a direct contractile effect can be unmasked by exposure to TTX. TTX is known to abolish almost all neural activity by blocking Na^+ channels. It also blocks neurotransmitter release (Kao, 1972). In the present study relaxation of duodenal circular muscles induced by CCK was inhibited completely by TTX under conditions where no contraction occurred. In contrast, the CCK-4-induced relaxation was suppressed only in part even by a high concentration of TTX. Relaxations in response to CCK-6, which has a lower affinity for CCK receptors and relatively lower intrinsic activity than CCK-8, were, like those with CCK-8, highly sensitive to TTX. Responses to CCK-5 showed a pattern intermediate between that for CCK-6 and CCK-4. In a previous paper (Kimura *et al.*, 1987), we showed different patterns of antagonism with the CCK antagonists, P2-II (D) and P3-I against CCK-8- and CCK-4-induced relaxation. The present results indicate that CCK-8 is completely TTX-sensitive, but that CCK-4 is able to invoke another mechanism for relaxation that is TTX-insensitive, probably by acting directly on muscle cells.

It is expected from the Goldman equation that high K^+ solutions would produce depolarization of the membrane and would produce contraction of the smooth muscle. In circular muscles of pig duo-

denum, however, it hyperpolarized the membrane and relaxed the muscle (Kimura *et al.*, 1984) as is reported for arterial muscles (Reiner, 1978), anococcygeus muscle (Ishii & Shimo, 1980) and taenia coli (Ishii & Shimo, 1979). The relaxation was concentration-dependent in a very narrow concentration range of K^+ . Reducing the Na^+ concentration to less than 20 mM depolarized the circular muscles of the guinea-pig stomach and contracture developed (Sakamoto & Tomita, 1982). However, low Na^+ solutions prepared by replacing NaCl with an osmotically equivalent amount of sucrose relaxed the muscles over a comparatively wide range of concentrations. This is somewhat different from the relaxing pattern of high K^+ . In a previous paper (Kimura *et al.*, 1984) we reported that both K^+ -induced or electrically stimulated relaxation was inhibited by TTX. Mesaconitine, which increases Na permeability (Catterall *et al.*, 1981), potentiated the three kinds of relaxation by CCK-8, high K^+ or electrical stimulation.

TEA also potentiated the three kinds of relaxation. TEA prolongs the action potential in nerves by preventing the delayed increase in K^+ permeability (Armstrong & Binstock, 1965), and it increases the liberation of a transmitter from the nerve as a result of an increased entry of Ca^{2+} (Gillespie & Tilmisany, 1976). Apamin was reported to inhibit the relaxation induced by noradrenaline and adenosine triphosphate in guinea-pig taenia coli (Weir & Weston, 1986). In the present study, apamin also potentiated the K^+ -induced relaxation, while it did not affect that produced by CCK-8. These results indicate that the mechanisms for relaxation by K^+ and CCK-8 are somewhat different and that the activation of the Ca^{2+} -activated K^+ -channel is not involved in CCK-8-induced relaxation. Ouabain, which potentiates the release of transmitters from nerves (Paton *et al.*, 1971; Bonaccorsi *et al.*, 1977), potentiated both relaxation induced by CCK-8 and K^+ (12.4–13.0 mM). Ouabain, mesaconitine and TEA had little effect on transmural and electrically stimulated relaxation; the reason for this is unknown.

In previous papers (Kimura *et al.*, 1983; 1984), we reported that the three kinds of treatment hyperpolarized the membrane. Two possible mechanisms are considered to explain the hyperpolarization: activation of the K^+ -channel and the Na^+ - K^+ -pump. CCK-8- and high K^+ -induced relaxations, however, were potentiated both by TEA or by ouabain. These results could not be explained in terms of direct action on the smooth muscles. The properties of the three kinds of relaxation are summarized in Table 1. It is postulated that all three agents producing relaxation involve the release of some inhibitory substances. The main difference among the three relaxants clearly concerns both CCK antagonist-

Table 1 The common properties of CCK-8, K⁺ and electrically stimulated relaxation in pig duodenal circular muscle

	CCK Antagonist	Na channel		K channel		Na ⁺ -K ⁺ pump	Resting membrane potential
		TTX	Mesaconitine	TEA	Apamin	Ouabain	
CCK-8	Inhibits	Inhibits	Potentiates	Potentiates	No effect	Potentiates	(Hyperpolarizes)*
K ⁺	No effect	Inhibits	Potentiates	Potentiates	Potentiates	Potentiates	Hyperpolarizes
Electrical	No effect	Inhibits	Potentiates	Potentiates	—	No effect	Hyperpolarizes

CCK-8 (0.263 nM), K⁺ (10.6–23.6 mM), electrical stimulation (3–3.5 V, 5 ms, 0.3–30 Hz for 20 s), CCK antagonist P3-I (168 μ M) and P2-II(D) (19.6 μ M), TTX (0.313 μ M), mesaconitine (0.158 μ M), TEA (0.597 mM), apamin (0.385–3.85 μ M), and ouabain (0.136 μ M).

* The effect of CCK-4 (Kimura *et al.*, 1983).

and Ca²⁺-dependent K⁺-channel antagonist-actions, as shown in Table 1.

CCK-8 relaxed the duodenal circular muscles by mechanisms clearly different from those for papaverine or isoprenaline, both of which were TTX-insensitive. VIP, which was proposed by Biancani *et al.* (1984) as a possible neurotransmitter candidate released by transmural stimulation in the lower oesophageal sphincter, relaxed the duodenal circular muscles and this effect was resistant to TTX.

In conclusion, CCK-8, K⁺, and transmural electrical field stimulation relaxed duodenal circular

muscles by releasing an endogenous inhibitory substance. The latter two mechanisms did not involve the release of CCK. The mechanisms for relaxation by CCK-4 are different from those of CCK-8.

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References

- ARMSTRONG, C.M. & BINSTOCK, L. (1965). Anomalous rectification in squid giant axon injected with tetraethylammonium chloride. *J. Gen. Physiol.*, **48**, 859–886.
- BEHAR, J. & BIANCANI, P. (1977). Effect of cholecystokinin-octapeptide on lower esophageal sphincter. *Gastroenterology*, **73**, 57–61.
- BEHAR, J. & BIANCANI, P. (1980). Effect of cholecystokinin and the octapeptide of cholecystokinin on the feline sphincter of Oddi and gallbladder. *J. Clin. Invest.*, **66**, 1231–1239.
- BIANCANI, P., WALSH, J.H. & BEHAR, J. (1984). Vasoactive intestinal polypeptide. A Neurotransmitter for lower esophageal sphincter relaxation. *J. Clin. Invest.*, **73**, 963–967.
- BONACCORSI, A., HERMSMEYER, K., SMITH, C.B. & BOHR, D.F. (1977). Norepinephrine release in isolated arteries induced by K-free solution. *Am. J. Physiol.*, **232**, H140–H145.
- CATTERALL, W.A., HARTSHORNE, R.P. & TAMKUN, M.M. (1981). Molecular pharmacology of voltage-sensitive sodium channels from mammalian brain. In *Topics in Molecular Pharmacology*, Vol. 1, ed. Burgen, A.S.V. & Roberts, G.C.K. pp. 222–245. Oxford: Elsevier.
- GILLESPIE, J.S. & TILMISANY, A.K. (1976). The action of tetraethylammonium chloride on the response of the rat anococcygeus muscle to motor and inhibitory nerve stimulation and to some drugs. *Br. J. Pharmacol.*, **58**, 47–55.
- ISHII, T. & SHIMO, Y. (1979). Potassium-induced relaxation of guinea-pig taenia coli. *Arch. Int. Pharmacodyn.*, **239**, 36–44.
- ISHII, T. & SHIMO, Y. (1980). Potassium-induced relaxation of the rat anococcygeus muscle. *Arch. Int. Pharmacodyn.*, **243**, 27–36.
- KAO, C.Y. (1972). Pharmacology of tetrodotoxin and saxitoxin. *Fed. Proc.*, **31**, 1117–1123.
- KIRKPATRICK, C.T. (1975). Excitation and contraction in bovine tracheal smooth muscle. *J. Physiol.*, **244**, 263–281.
- KIMURA, I., KIMURA, M. & KIMURA, M. (1984). Potassium induces relaxation and hyperpolarization of circular muscles but contraction of longitudinal muscles of pig duodenum. *Br. J. Pharmacol.*, **83**, 187–193.
- KIMURA, I., KONDOH, T. & KIMURA, M. (1987). Proglumide-peptides and CCK-antagonistic action in hog duodenal circular muscle. *Eur. J. Pharmacol.*, **134**, 181–187.
- KIMURA, I., KIMURA, M., NAKAYAMA, N. & KIMURA, M. (1983). Relaxation and Ca-spike suppression in circular and longitudinal muscles of hog bile duct ampulla by CCK-C-terminal peptides. *Arch. Int. Pharmacodyn.*, **265**, 320–334.
- PATON, W.D.M., VIZI, E.S. & ABOO ZAR, M. (1971). The mechanism of acetylcholine release from parasympathetic nerves. *J. Physiol.*, **215**, 819–848.
- REINER, O. (1978). The role of the electrogenic sodium

- pump in the potassium relaxation of the rabbit ear artery. *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **303**, 213-220.
- SAKAMOTO, Y. & TOMITA, T. (1982). Depolarization produced by sodium removal in the circular muscle of the guinea-pig stomach. *J. Physiol.*, **326**, 329-339.
- SANDBLOM, P., VOEGTLIN, W.L. & IVY, A.C. (1935). The effect of cholecystokinin on the choledochoduodenal mechanism (sphincter of Oddi). *Am. J. Physiol.*, **93**, 175-180.
- STURDEVANT, R.A.L. & KUN, T. (1974). Interaction of pentagastrin and the octapeptide of cholecystokinin on the human lower esophageal sphincter. *Gut*, **15**, 700-702.
- WEIR, S.W. & WESTON, A.H. (1986). Effect of apamin on responses to BRL 34915, nicorandil and other relaxants in the guinea-pig taenia caeci. *Br. J. Pharmacol.*, **88**, 113-120.

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