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Modification by charybdotoxin and apamin of spontaneous electrical and mechanical activity of the circular smooth muscle of the guinea-pig stomach

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1 The effects of charybdotoxin and apamin, putative blockers of Ca^{2+}-activated K^+ channels, on spontaneous electrical and mechanical activity of circular smooth muscle of the guinea-pig stomach antrum were examined in the presence of 1 μM tetrodotoxin and 1 μM atropine.

2 Both charybdotoxin (> 3 nM) and apamin (> 3 nM) dose-dependently increased the amplitude of spontaneous contractions without altering their frequency. The maximum effect of charybdotoxin was much greater than that of apamin. Both toxins increased the amplitude of intracellular Ca^{2+} oscillations measured with fura-2.

3 When the extracellular Ca^{2+} concentration was lowered to 1.5 mM or less, apamin did not significantly potentiate the contractions whereas charybdotoxin still potentiated them but with less potency.

4 Charybdotoxin (30 nM) increased the amplitude of spikes and slow waves, and slightly decreased the resting membrane potential. On the other hand, apamin (100 nM) preferentially increased the slow wave amplitude with no effect on the resting membrane potential.

5 These results suggest that both toxins affect the spontaneous contraction by modifying the electrical activity and that charybdotoxin-sensitive K^+ channels and apamin-sensitive ones are differently involved in the spontaneous electrical activity.

Keywords: Charybdotoxin; apamin; guinea-pig stomach; membrane potential; contraction; K channel; slow wave

Introduction

Charybdotoxin (CTX), a component of the venom of the scorpion *Leiurus quinquestriatus*, is a 37 amino acid peptide with three disulphide bridges (Sugg *et al.*, 1990). It was first introduced as a selective blocker of large conductance Ca^{2+}-activated K^+ channels (BK channels) in skeletal muscle (Miller *et al.*, 1985). The blocking action of CTX on Ca^{2+}-activated K^+ channels in smooth muscles has been demonstrated in myocytes from the rabbit aorta (Talvenheimo *et al.*, 1988) and from the canine colon and stomach (Carl *et al.*, 1990a). CTX caused or potentiated contractions in many types of smooth muscles (Murray *et al.*, 1991; Suarez-Kurtz *et al.*, 1991; Ito *et al.*, 1992). Apamin is a bee venom polypeptide of 18 amino acids with two disulphide bridges (Romey *et al.*, 1984), which has been shown to block a Ca^{2+}-activated K^+ channel of small conductance (SK channel) in skeletal muscle (Blatz & Magleby, 1986). This channel may be responsible for the generation of after-hyperpolarization in some nerves (Romey *et al.*, 1984). In some types of visceral smooth muscle this toxin reversed the ATP-induced hyperpolarization to depolarization or blocked inhibitory junction potentials (Vladimirova & Shuba, 1978; Banks *et al.*, 1979; Maas & Den Hertog, 1979; Komori & Suzuki, 1986), whereas such effects were not found in other muscles (Nakao *et al.*, 1986; Lydrup, 1991). The lack of a direct contractile action of apamin has been observed in many kinds of smooth muscles (Allen *et al.*, 1986; Winquist *et al.*, 1989; Fujii *et al.*, 1990), although contraction is initiated in a few tissues (e.g. the guinea-pig taenia coli, Maas & Den Hertog, 1979). These two toxins are becoming specific tools for the study of the role of Ca^{2+}-activated K^+ channels in excitable and non-excitable cells.

Circular smooth muscle of the smooth antrum exhibits spontaneous electrical changes, which are composed of slow waves and superimposed spikes (Tomita, 1981). The involvement of Ca^{2+}-activated K^+ channels in such spontaneous activity has been suggested (Mitra & Morad, 1985; Carl *et al.*, 1990b). In this study we examined the effects of apamin and CTX on the spontaneous electrical and mechanical activity of the guinea-pig stomach antrum in order to ascertain whether some kinds of Ca^{2+}-activated K^+ channels are involved.

Methods

The methods were those described in a previous paper (Ito *et al.*, 1992) except for the measurement of Ca^{2+} transients with fura-2. The stomach antra were isolated from male guinea-pigs (200–250 g). After removing the mucosal layer, small strips of circular muscle with the longitudinal layer attached were cut to 2 mm wide and 10 mm long for tension or fura-2 experiments, or 2 mm wide and 4 mm long for microelectrode experiments. For tension experiments the muscle was suspended along the direction of the circular muscle with a basal tension of 1 g in a Magnus bath containing 5 ml Krebs solution (37°C) gassed with 95% O_2 and 5% CO_2. Isometric tension was recorded with a force-displacement transducer (Nihon-Kohden SB-1T).

Cytosolic Ca^{2+} was measured with fura-2 simultaneously with tension as described by Sato *et al.* (1988). Briefly, the muscle was loaded with 5 μM fura-2/AM that had been sonicated together with 0.02% cremophore EL to increase the solubility of fura-2/AM. After loading for 4–5 h at room temperature, the preparation was placed horizontally in an organ bath constructed in a fluorimeter (JASCO CAF-100).

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One end of the muscle was connected to a force-displacement transducer. A part of the muscle was excited by light through rotating filters (48 Hz) of 340 nm and 380 nm. The emitted light from the muscle was collected by a photomultiplier, and the ratio of fluorescence at 500 nm due to excitation at 340 nm and 380 nm was calculated. The time constant for the signal was set at 0.25 s.

Preparations used for microelectrode studies were mounted in a superfusion chamber (fluid volume 2 ml) and superfused with the Krebs solution (35°C) gassed with 95% O_2 and 5% CO_2. A cell was impaled from the mucosal side by a glass microelectrode filled with 3 M KCl (tip resistance 50–80 MΩ). The signal was amplified by a microelectrode amplifier (Nihon-Kohden MEZ-8101) and recorded on a pen-writing oscillograph (NEC-Sa'nei Recti-Horiz) and video cassette tape using a PCM processor (Sony PCM-501 ES). The effects of apamin and CTX were observed in the presence of 1 μM tetrodotoxin and 1 μM atropine.

The Krebs solution had the following composition (mM): NaCl 137.4, KCl 5.9, $CaCl_2$ 2.5, $MgCl_2$ 1.2, $NaHCO_3$ 15.5, NaH_2PO_4 1.2 and glucose 11.5 (pH 7.4 when gassed with 5% CO_2). Drugs used were apamin (Sigma), charybdotoxin (CTX, Peptide Institute), cromakalim (Beecham), atropine (Tokyo Kasei) tetrodotoxin (Sankyo), acetylcholine (Tokyo Kasei), fura-2/AM (dissolved in dimethyl sulphoxide, Dojin Laboratories) and cremophore EL (Nacalai Tesque). Cromakalim was dissolved in ethanol as described elsewhere (Ito *et al.*, 1992). Other drugs were dissolved in distilled water.

Data are expressed as mean ± s.e.mean. The pD_2 value was calculated as described by Ito *et al.* (1992). The statistical significance was tested by Student's *t* test for paired or unpaired observations at the level of $P<0.05$.

Results

Effects of charybdotoxin and apamin on the spontaneous contraction

Figure 1 shows examples of the effects of CTX (10 nM and 30 nM) and apamin (10 nM and 100 nM) on spontaneous contractions of circular smooth muscle of the guinea-pig stomach antrum. Both toxins increased the amplitude of the spontaneous contractions without affecting their frequency. With high concentrations of CTX (100 nM or more) a slight elevation of the resting tension was observed in some muscles (e.g., in 3 of 9 preparations at 100 nM), while such an effect was rarely observed in the case of apamin. The presence of tetrodotoxin (1 μM) and atropine (1 μM) did not affect the actions of CTX and apamin, suggesting that the potentiation was not mediated by neural factors. The enhancement of contraction by CTX and apamin was dependent on the concentration (Figure 2), and also on the spontaneous contraction itself to some extent, in that, when the spontaneous contraction was small, the potentiation tended to be relatively small. Although the effect of apamin was the same at more than 10 nM, the time to peak effect was shorter with a higher concentration. The effects of both toxins were reversible if more than 1 h washing was given. The maximal potentiation of spontaneous contraction by CTX (486 ± 131% of control contraction, at 100 nM, $n=9$) was much greater ($P<0.05$, unpaired *t* test) than that by apamin (246 ± 18% at 10 nM, $n=37$).

When cromakalim was cumulatively added during the sustained enhancement due to 30 nM CTX or 100 nM apamin, it inhibited the contraction with a pD_2 of 6.57 ± 0.05 ($n=7$) or 6.57 ± 0.09 ($n=10$), respectively (data not shown). Cromakalim inhibited the spontaneous contraction in the absence of either toxin with a pD_2 of 6.65 ± 0.06 ($n=9$, Ito *et al.*, 1992).

Relations of mechanical effects of charybdotoxin and apamin to Ca^{2+}

Cytosolic Ca^{2+} in gastric smooth muscles was measured with the Ca^{2+} indicator fura-2 simultaneously with the tension. As shown in Figure 3, cytosolic Ca^{2+} oscillated spontaneously, accompanied by spontaneous contractions. Acetylcholine (1 μM) elevated the basal level of Ca^{2+} with superimposing oscillations, resulting in development of basal tension. CTX (30 nM) increased the amplitude of Ca^{2+} oscillation and contraction to 151.4 ± 10.4% and 321.7 ± 30.1% ($n=23$), re-

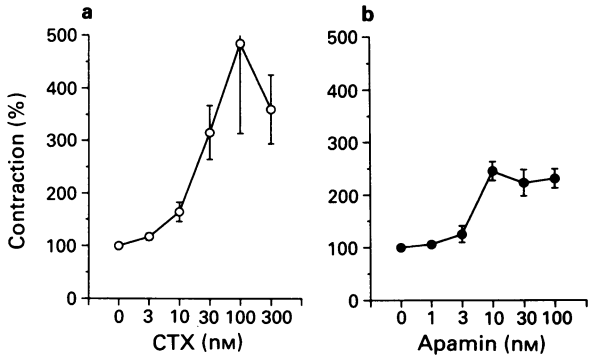

Figure 2 Dose-response relationship for charybdotoxin (CTX)- and apamin-induced potentiations of spontaneous contraction of the guinea-pig stomach. On the ordinate scale the amplitude of spontaneous contraction just prior to application of CTX or apamin is expressed as 100%. The points are means with the s.e.mean of 7–22 preparations for CTX and 13–57 preparations for apamin.

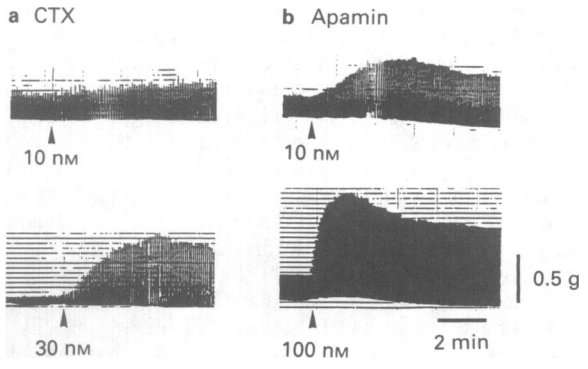

Figure 1 Examples of the potentiating effects of charybdotoxin (CTX, 10 nM and 30 nM) and apamin (10 nM and 100 nM) on spontaneous contractions in circular smooth muscles of the guinea-pig stomach.

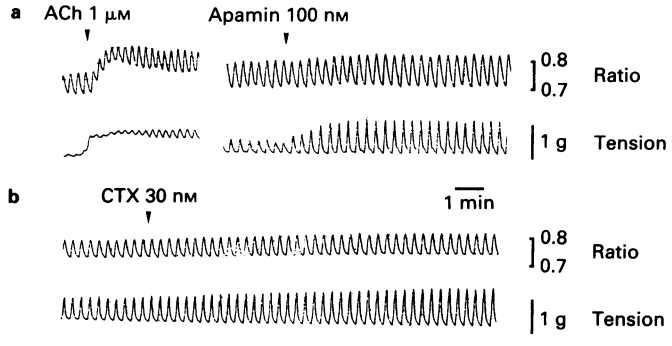

Figure 3 Examples of the effects of acetylcholine (ACh), apamin and charybdotoxin (CTX) on cytosolic Ca^{2+} levels and tension in the guinea-pig stomach loaded with fura-2. (a) Effects of acetylcholine (1 μM) and apamin (100 nM). The data were obtained from the same preparation. (b) Effects of CTX (30 nM). In (a) and (b), upper trace: fluorescence ratio, lower trace: tension.

spectively, of the control level. In some muscles, CTX slightly elevated the basal level of Ca^{2+}, but the elevation was much smaller than that due to acetylcholine. Apamin (100 nM) also increased the parameters to 134.9 ± 4.9% and 196.7 ± 18.5% ($n = 14$), respectively.

The spontaneous contraction of the guinea-pig stomach depended on the concentration of external Ca^{2+}. With decreased Ca^{2+} concentrations the amplitude of spontaneous contractions decreased. The frequency of spontaneous contraction did not change until Ca^{2+} was reduced to 0.5 mM when it decreased. Figure 4 shows the potentiating effects of 30 nM CTX and 100 nM apamin at various concentrations of external Ca^{2+}. When external Ca^{2+} was 1.5 mM or less, apamin did not significantly potentiate the contraction. With lower Ca^{2+} concentrations the potentiating effect of CTX decreased but was still significant.

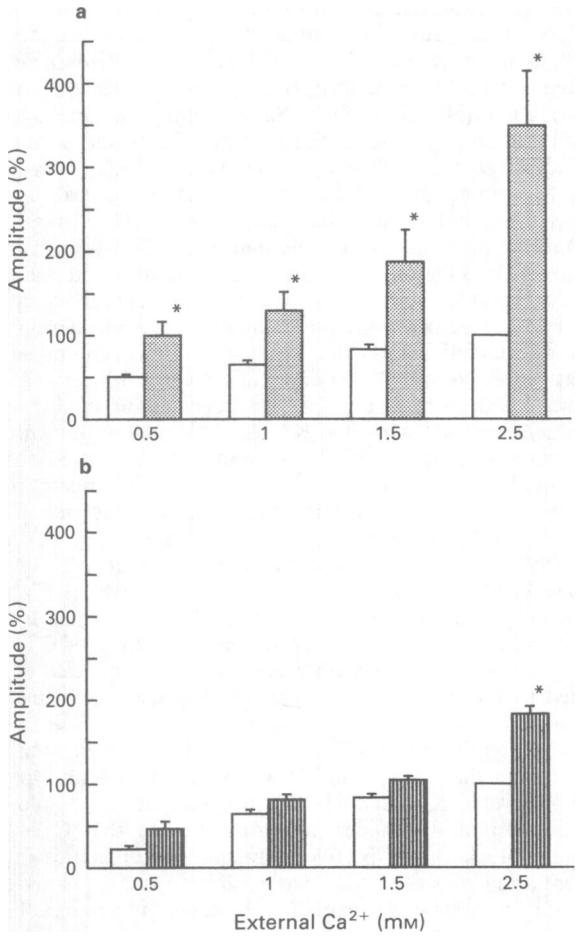

Figure 4 Dependence of potentiation by charybdotoxin (CTX, 30 nM) and apamin (100 nM) of spontaneous contraction on external Ca^{2+} concentration. The amplitude of control spontaneous contraction at 2.5 mM $[Ca^{2+}]_o$ is expressed as 100%. In (a) open columns: control; shaded columns CTX. In (b) open columns: control; shaded columns apamin. *Significantly different ($P < 0.05$) from control at the respective $[Ca^{2+}]_o$ (paired t test). Data represent the mean with the s.e.mean of 8–10 preparations for CTX or 10–20 preparations for apamin.

Effects of charybdotoxin and apamin on the membrane potential

Every cell in the antrum region impaled with a microelectrode exhibited slow waves with a frequency of 4–8 cycles min^{-1}. However, not every cell showed spike activity on the slow waves. Figures 5 and 6 show examples of the effects of 30 nM CTX and 100 nM apamin, respectively, on the membrane potential of circular smooth muscles with or without spontaneously generated spikes, and Table 1 summarizes the resulting changes in membrane potential parameters. When

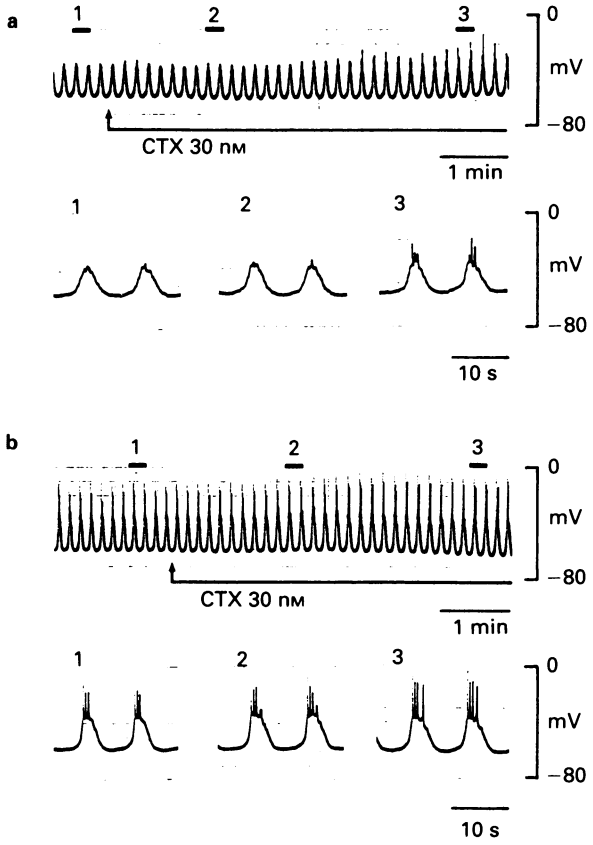

Figure 5 Effect of 30 nM charybdotoxin (CTX) on the membrane potential of guinea-pig stomach. (a) Effects of CTX in a preparation which did not exhibit spikes on the slow waves. (b) Effects in a preparation exhibiting spikes on the slow waves. In (a) and (b) the lower panels show the recordings of the sections labelled 1, 2 and 3 in the upper panel at a faster time base.

Table 1 Changes in electrical properties of guinea-pig stomach caused by charybdotoxin (CTX) and apamin

Treatment	*Resting membrane potential* (mV)	*Slow wave amplitude* (mV)	*Slow wave frequency* (min^{-1})	*Spike amplitude* (mV)
Control	− 68.3 ± 2.3	20.3 ± 3.4	5.7 ± 0.5	5.0 ± 2.4
+ CTX 10 nM	− 63.4 ± 3.8*	20.8 ± 2.8	6.1 ± 0.3	8.1 ± 2.5*
Control	− 61.0 ± 4.3	20.2 ± 2.7	6.2 ± 0.5	3.6 ± 1.4
+ CTX 30 nM	− 56.6 ± 4.6*	22.7 ± 3.2*	6.2 ± 0.5	5.9 ± 0.5*
Control	− 59.8 ± 1.4	21.0 ± 1.5	6.2 ± 0.4	4.2 ± 1.8
+ Apamin 100 nM	− 58.9 ± 1.6	25.2 ± 1.2*	6.2 ± 0.4	6.3 ± 0.4

*$P < 0.05$ (vs control, paired t test), $n = 6$ for each group. Data are collected from the preparations which exhibited spike activity on slow waves.

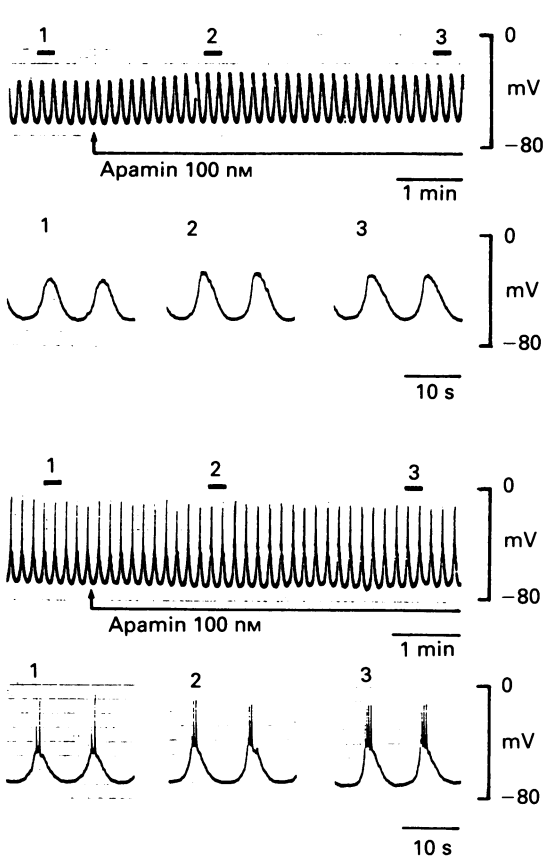

Figure 6 Effect of 100 nM apamin on the membrane potential of guinea-pig stomach. (a) Effects of apamin in a preparation which did not exhibit spikes on the slow waves. (b) Effects in a preparation exhibiting spikes on the slow waves. In (a) and (b) the lower panels show the recordings of the sections labelled 1, 2 and 3 in the upper panel at a faster time base.

CTX was applied to cells not showing spikes, it often induced spike activity, concomitant with a slight increase in slow wave amplitude (Figure 5a). In cells showing spikes, CTX increased the amplitude and/or the number of spikes (Figure 5b). In many cells CTX decreased the resting membrane potential (maximum diastolic potential) by 3–5 mV. Lower concentration of CTX (10 nM) increased the amplitude of spikes with no effect on slow waves (Table 1).

On the other hand, apamin increased the amplitude of slow waves in either spike-generating or non-generating cells (Figure 6). No special effects on the spikes were observed (Table 1). Apamin did not alter the resting membrane potential. When a 10 times higher concentration of apamin (1 μM) was used, the effects were the same as with the 100 nM apamin (3 observations, data not shown).

Discussion

In this study CTX and apamin enhanced the spontaneous contractions of the circular smooth muscle of the guinea-pig stomach. This enhancement resulted from an increase in the amplitude of intracellular Ca^{2+} oscillations. Observations showing that apamin did cause or potentiate contraction in smooth muscles under physiological conditions are very rare (Maas & Den Hertog, 1979). This indicates that apamin-sensitive K^+ channels are not functional in most types of smooth muscle and the guinea-pig stomach is a rare example of a tissue in which these channels are involved in spontaneous activity. The fact that apamin inhibited ATP-induced hyperpolarization and the inhibitory junction potential in the guinea-pig stomach (Vladimirova & Shuba, 1978; Komori & Suzuki, 1986) supports the view that apamin-sensitive K^+ channels exist in this tissue.

Although small conductance Ca^{2+}-activated K^+ channels have been found in the rabbit portal vein (Inoue *et al.*, 1985), taenia coli (Hu *et al.*, 1989) and jejunum, and the guinea-pig mesenteric artery (Benham *et al.*, 1986), the channel conductance (63–92 pS) is still larger than that of the apamin-sensitive SK channel found in skeletal muscle (10–14 pS, Blatz & Magleby, 1986), and the sensitivity to apamin was not tested in smooth muscles. Therefore, the existence of Ca^{2+}-activated K^+ channels which are sensitive to apamin should be demonstrated by a patch clamp study. Since apamin antagonized responses to putative inhibitory transmitters in the guinea-pig stomach (Vladimirova & Shuba, 1978; Komori & Suzuki, 1986), taenia coli (Vladimirova & Shuba, 1978; Maas & Den Hertog, 1979; Wier & Weston, 1986), colon (Hugues *et al.*, 1982) or ileum (Yamanaka *et al.*, 1985) but showed no action in vascular smooth muscles (Winquist *et al.*, 1989; Brayden & Nelson, 1992), it is likely that apamin-sensitive K^+ channels exist in visceral smooth muscles but not in vascular smooth muscles.

On the other hand, the existence of CTX-sensitive K^+ channels is suggested in both non-vascular and vascular smooth muscles since CTX caused or potentiated contractions of the guinea-pig bladder, taenia coli, portal vein and uterus (Suarez-Kurtz *et al.*, 1991), the rabbit cerebral artery (Brayden & Nelson, 1992) and the rat carotid artery (Asano *et al.*, 1992). Although CTX was reported to inhibit small conductance Ca^{2+}-activated K^+ channels in *Aplysia* neurones (Herman & Erxleben, 1987) or voltage-dependent K^+ channels in T-lymphocytes (Deutsch *et al.*, 1991), many patch clamp studies have confirmed that large conductance Ca^{2+}-activated K^+ channels are sensitive to CTX e.g. in single smooth muscle cells from stomach (Carl *et al.*, 1990a), trachealis (Murray *et al.*, 1991) and vascular tissues (Talvenheimo *et al.*, 1988; Pavenstadt *et al.*, 1991; Brayden & Nelson, 1992). Therefore, it is likely that the effects of CTX on the guinea-pig stomach observed in this study can be ascribed to the block of large conductance Ca^{2+}-activated K^+ channels.

The potentiation by CTX of spontaneous contraction was larger than that by apamin. If we assume that both types of Ca^{2+}-activated K^+ channel are functioning in a physiological contraction of the guinea-pig stomach, and that CTX and apamin block the respective channels, the difference in the potentiating effects of the two toxins on spontaneous contractions may be easily explained by the conductances. If the Ca^{2+} concentration of the external medium ($[Ca^{2+}]_o$) was lowered, the cytoplasmic Ca^{2+} concentration would be expected to decrease, and the activity of Ca^{2+}-activated K^+ channels will be reduced. The reduced potentiation by CTX and apamin at low $[Ca^{2+}]_o$ indicates that the action of these toxins is associated with Ca^{2+}-activated K^+ channels. Apamin did not significantly increase the contraction at 1.5 mM Ca^{2+} or less, whereas, CTX significantly potentiated it even at 0.5 mM Ca^{2+}. The small conductance Ca^{2+}-activated K^+ channel in skeletal muscles is more sensitive to Ca^{2+} than the large conductance channel (Blatz & Magleby, 1986). If the apamin-sensitive K^+ channel in the guinea-pig stomach is also more sensitive to Ca^{2+} than the CTX-sensitive one, the apamin-induced potentiation of contractions should have been observed at lower Ca^{2+} concentrations than for the CTX-effect. The significant potentiation by CTX but not by apamin at low Ca^{2+} concentrations appears contradictory. However, the conductance of BK channels is 5–10 times larger than that of SK channels (Cook, 1988). Therefore, an alternative explanation for the lack of potentiation at low Ca^{2+} concentration by apamin could be because of the small conductance of the channel.

The effects of CTX and apamin on the membrane potential were tested at 30 nM and 100 nM which enhanced spontaneous contractions by 316 ± 52% ($n = 22$) and 231 ± 18%

($n = 57$), respectively. CTX and apamin had different effects on the electrical activity of guinea-pig stomach. The predominant effect of apamin was an increase in the slow wave amplitude, while CTX increased the amplitudes of both spikes and slow waves. At a lower concentration (10 nM) CTX affected the amplitude of the spikes but not the slow waves. The increase in slow wave amplitude by apamin was greater than that by CTX. Some studies suggest that large conductance Ca^{2+}-activated K^+ channels show voltage-dependency, such that the open probability of the channel increases at a less negative voltage (Benham *et al.*, 1986; Singer & Walsh, 1987; Toro *et al.*, 1991). Therefore, it is possible that large conductance Ca^{2+}-activated K^+ channels play a more significant role in the spike phase than the slow wave phase, which would account for the fact that the CTX-effect appears more noticeably on spikes. On the assumption that the CTX-sensitive K^+ channel has a large conductance, the blockade by CTX of such a channel would remove the large offsetting effect of Ca^{2+}-activated K^+ channels on Ca^{2+} channels, thus permitting greater Ca^{2+} entry and resulting in a considerable potentiation of contraction.

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