Br. J. Pharmacol. (1992), **105**, 335–340 © Macmillan Press Ltd, 1992

Effects of cromakalim on the contraction and the membrane potential of the circular smooth muscle of guinea-pig stomach

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1 The effects of cromakalim on mechanical and electrical activities of the circular smooth muscles of guinea-pig stomach antrum were observed.

2 Cromakalim ($>1 \times 10^{-7}$ M) decreased the amplitude of spontaneous rhythmic contractions and also the acetylcholine-enhanced spontaneous contractions. Cromakalim was less effective against the 25.9 mM and 35.9 mM K^+-induced tonic contractions.

3 Glibenclamide (1×10^{-6} M) itself caused no detectable change in the spontaneous contractions, those potentiated by acetylcholine or tonic contractions induced by high K^+ solutions, but attenuated the actions of cromakalim. On the other hand, charybdotoxin (3×10^{-8} M) increased the amplitude of spontaneous contractions but failed to affect the actions of cromakalim.

4 Cromakalim ($>1 \times 10^{-6}$ M) decreased the amplitude and duration of slow waves, and hyperpolarized the membrane. These actions of cromakalim were completely antagonized by 1×10^{-6} M glibenclamide, whereas part of the effects of cromakalim on mechanical activity was resistant to glibenclamide.

5 The results suggest that the inhibition by cromakalim of the electrical activity and the hyperpolarization, which may be associated with the opening of glibenclamide-sensitive K^+ channel, are responsible for its inhibitory action on circular smooth muscle of guinea-pig stomach. Further, some effects independent of glibenclamide-sensitive K^+ channel may also be responsible for the mechanical effect.

Keywords: Cromakalim; guinea-pig stomach; membrane potential; contraction; glibenclamide; charybdotoxin; K channel

Introduction

It has been postulated that cromakalim relaxes many types of smooth muscle by hyperpolarizing the membrane through activation of K^+ channels (Hamilton *et al.*, 1986; Allen *et al.*, 1986; Hollingsworth *et al.*, 1987; Standen *et al.*, 1989). The nature of cromakalim as a 'K^+ channel opener' was confirmed not only by electrophysiological experiments but also by $^{86}Rb^+$ efflux experiments (Hamilton *et al.*, 1986; Weir & Weston, 1986; Standen *et al.*, 1989; Masuzawa *et al.*, 1990a,b). Despite its classification as a K^+ channel opener, it is questionable whether its relaxing action on smooth muscles is solely due to membrane hyperpolarization, since the threshold concentration required for muscle relaxation is usually lower than that required for the hyperpolarization or for the stimulation of $^{86}Rb^+$ or $^{42}K^+$ efflux (Hamilton *et al.*, 1986; Hollingsworth *et al.*, 1987; Quast, 1987; Shetty & Weiss, 1987; Gillespie & Sheng, 1988). Such discrepancies are prominent mainly in spontaneously active muscles such as portal vein (Hamilton *et al.*, 1986), uterus (Hollingsworth *et al.*, 1987), trachea (Allen *et al.*, 1986) or urinary bladder (Foster *et al.*, 1989).

We have now investigated the effects of cromakalim on the mechanical and electrical properties of circular smooth muscle of the guinea-pig stomach. This smooth muscle exhibits two types of spontaneous electrical activity, composing slow waves and spike potentials (Tomita, 1981). Tomita & Brading (1990) briefly reported that cromakalim inhibited gastric mechanical activity with no significant effects on the electrical activity of this tissue. The objective of the present study was, therefore, to study further the relationship between electrical and mechanical responses during cromakalim-induced relaxations.

The effects of two types of K^+ channel blocker, glibenclamide and charybdotoxin, on the actions of cromakalim were also investigated, to determine the types of K^+ channel involved in the actions of cromakalim. Putatively glibenclamide inhibits ATP-sensitive K^+ channels (Schmid-Antomarchi *et al.*, 1987; Standen *et al.*, 1989), whereas charybdotoxin blocks the Ca^{2+}-activated K^+ channels (Miller *et al.*, 1985; Talvenheimo *et al.*, 1988; Carl *et al.*, 1990a), although the selectivity of these agents for particular smooth muscle K^+ channels has not been determined.

Methods

Adult male guinea-pigs (200–250 g) were stunned and bled. Small strips of circular muscle were dissected from the antral region of the stomach as described by Ono & Suzuki (1987).

For microelectrode studies the preparation (2 mm wide, 4 mm long) was mounted in a superfusion chamber (fluid volume about 2 ml) with tiny insect pins and superfused with the Krebs solution (35°C) gassed with 95% O_2 and 5% CO_2 at a flow rate of 2 ml min^{-1}. The membrane potential was measured with a glass microelectrode of a tip resistance 40–80 mΩ when filled with 3 M KCl, and the electrode was inserted into a cell from the mucosal side. The signal was amplified by a microelectrode amplifier (Nihon-Kohden MEZ-8101), displayed on an oscilloscope (Nihon-Kohden VC-9) and recorded on a pen-writing oscillograph (NEC-Sa'nei Recti-Horiz) and video cassette tape using a PCM processor (Sony PCM-501 ES). For tension recording the preparation (2 mm wide, 10 mm long) was suspended in a vertical position in a Magnus bath containing 5 ml Krebs solution (37°C) gassed with 95% O_2 and 5% CO_2, with the upper end of the muscle

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connected to an isometric transducer (Nihon-Kohden SB-1T). In some experiments one end of a preparation (2 mm wide, 4 mm long) was fixed in the superfusion chamber for the microelectrode study with insect pins and the other end was connected to a transducer in a horizontal direction. A basal tension of 1 g was maintained throughout the tension experiment.

The Krebs solution used had the following composition (mM): Na^+ 137.4, K^+ 5.9, Ca^{2+} 2.5, Mg^{2+} 1.2, HCO_3^- 15.5, $H_2PO_4^-$ 1.2, Cl^- 134.0, glucose 11.5 (pH 7.4). The high K^+ solution was hypertonic and prepared by adding KCl to the Krebs solution to give a final concentration of 25.9 mM or 35.9 mM K^+. Drugs used were cromakalim (Beecham), glibenclamide (Sigma), charybdotoxin (Peptide Institute), acetylcholine (ACh, Nakarai Chemicals), tetrodotoxin (Sankyo) and atropine (Tokyo Kasei). Cromakalim and glibenclamide were dissolved in ethanol as described elsewhere (Masuzawa *et al.*, 1990a). Other substances were dissolved in distilled water.

Data are expressed as mean ± s.e.mean. To determine the pD_2 value of cromakalim, the EC_{50} was calculated from the log dose-response relationship in each muscle as the concentration required to inhibit a contraction to 50% of the control level and the mean ± s.e.mean of its negative log (pD_2) was calculated. When the antagonism of glibenclamide to cromakalim-induced inhibition of mechanical activity was tested, the control response to cromakalim was observed and after 1 h rinsing of muscles the response was again observed in the presence of glibenclamide. Statistical significance was assessed for responses before and after glibenclamide by Student's *t* test (paired *t* test).

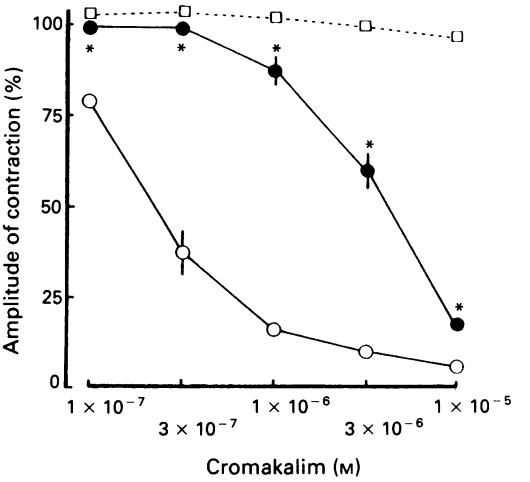

Figure 1 The effect of cromakalim on spontaneous contractions of the circular smooth muscle of guinea-pig stomach in the absence (○) and presence (●) of glibenclamide 1×10^{-6} M. Ordinate scale: 100% = the amplitude of the spontaneous contractions prior to application of the drugs. When used, glibenclamide was applied 10 min before the addition of cromakalim. Broken line represents the effect of vehicle (ethanol), which was added at the same time intervals as cromakalim. Data represent the means with the s.e.means (vertical lines) of 9 experiments. The effect of glibenclamide plus cromakalim was significantly different from cromakalim alone (* $P < 0.05$, paired *t* test).

Results

Effects of cromakalim on the mechanical activity

Circular smooth muscles of the guinea-pig stomach antrum exhibited spontaneous rhythmic contractions under a basal tension of 1 g. When applied cumulatively, cromakalim (1×10^{-7}–1×10^{-5} M) decreased the amplitude of spontaneous contractions in a concentration-dependent manner with a PD_2 of 6.65 ± 0.06 ($n = 9$, Figure 1). However, cromakalim did not significantly affect the frequency of the spontaneous contractions as long as the contractions could be observed, i.e. the frequency was 4.3 ± 1.8 contractions min^{-1} before the application of cromakalim whereas it was 4.5 ± 1.3 contractions min^{-1} in the presence of 3×10^{-6} M cromakalim. In a different group of muscles the effect of ethanol, which was used to dissolve cromakalim and glibenclamide, was observed. Ethanol in concentrations which were the same as those added with cromakalim did not affect the spontaneous contractions (Figure 1). This result also shows that spontaneous contractions remained constant over the time course of the experiment.

When 1×10^{-6} M ACh was applied, the basal tension was elevated (tonic contraction) following a transient large contraction and the spontaneous contractions were augmented to 990 ± 155% ($n = 8$) of the predrug level. Addition of cromakalim decreased the amplitude of enhanced spontaneous contractions and the tonic contraction with a PD_2 of 6.38 ± 0.08 and 6.27 ± 0.20 ($n = 8$), respectively (Figure 2). The vehicle (ethanol) for cromakalim, which was added with the same interval as cromakalim, did not affect the ACh-enhanced contractions over the time course of the experiment. Addition of K^+ (final $[K^+]_o = 25.9$ mM and 35.9 mM) elevated the basal tension which consisted of a transient component followed by a sustained one. The tonic contraction was obviously larger in 35.9 mM $[K^+]_o$ than in 25.9 mM $[K^+]_o$. Often in the presence of 25.9 mM K^+, the augmented spontaneous contractions were superimposed on the tonic response, the frequency of tension waves was not different from that in normal $[K^+]_o$. With

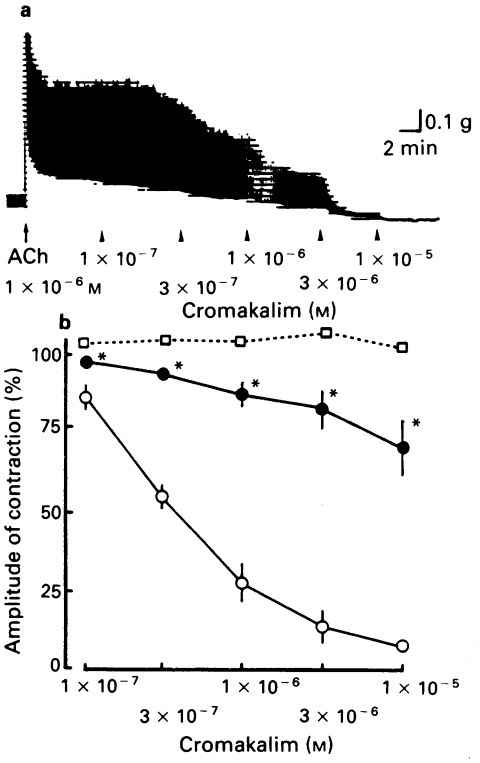

Figure 2 An example of the effect of cumulatively added cromakalim on the acetylcholine (ACh)-enhanced spontaneous contractions of the guinea-pig stomach (a) and the summarized dose-effect relationship of cromakalim in the absence (○) and presence (●) of glibenclamide 1×10^{-6} M (b). When the contraction reached the steady state after the addition of ACh 1×10^{-6} M cromakalim was added. In (b) 100% on the ordinate scale represents the amplitude of the enhanced spontaneous contraction just before the application of cromakalim. Broken line represents the effect of vehicle (ethanol), which was added at the same time intervals as cromakalim. When used, glibenclamide was applied 10 min before the addition of ACh. Data represent the means with the s.e.mean (vertical lines) of 8 experiments. The effect of glibenclamide plus cromakalim was significantly different from cromakalim alone (* $P < 0.05$, paired *t* test).

35.9 mM K^+, the spontaneous contractions were abolished. Cromakalim added during the sustained phase of the tonic contraction relaxed the muscle with a pD_2 of 5.89 ± 0.17 ($n = 8$) and 5.43 ± 0.21 ($n = 7$) at 25.9 mM and 35.9 mM K^+, respectively (Figure 3). These pD_2 values were significantly smaller than the value observed for the spontaneous contractions in normal K^+ medium ($P < 0.01$).

Effects of cromakalim on the electrical activity

Circular smooth muscles of the antrum region generated spontaneous electrical activity, which consisted of slow waves with superimposed spike potentials (Tomita, 1981), events which were accompanied by phasic contractions. Figure 4 shows an example of the change of membrane potential caused by 1×10^{-6} M cromakalim. In this tissue cromakalim inhibited spontaneous electrical and mechanical activities with a relatively slow time course. Two min after superfusion with cromakalim the amplitude of both slow waves and spike potentials decreased, with a substantial hyperpolarization of the membrane. After 3 min exposure, the membrane was further hyperpolarized, the amplitude of slow waves decreased to below 30% of control and spike activity was abolished, with no significant change in slow wave frequency (Figure 4). These effects of cromakalim are summarized in Figure 5.

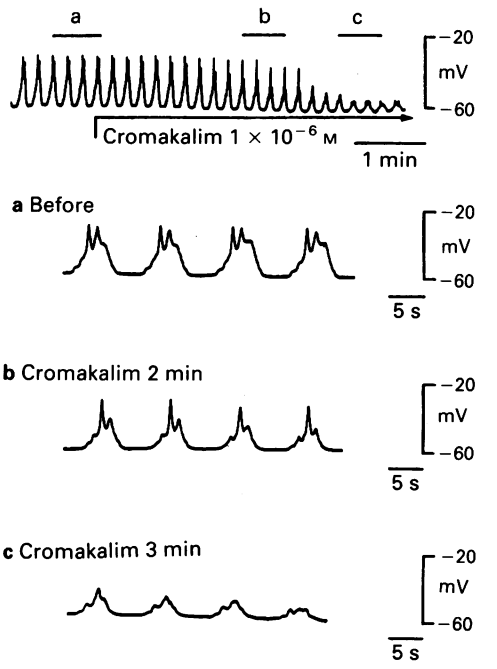

Figure 4 An example of the effect of cromakalim 1×10^{-6} M on the membrane potential of the circular smooth muscle of guinea-pig stomach. Lower three traces; records of membrane potential on an expanded time scale, which correspond to before, 2 min and 3 min after the superfusion of cromakalim shown in the top trace.

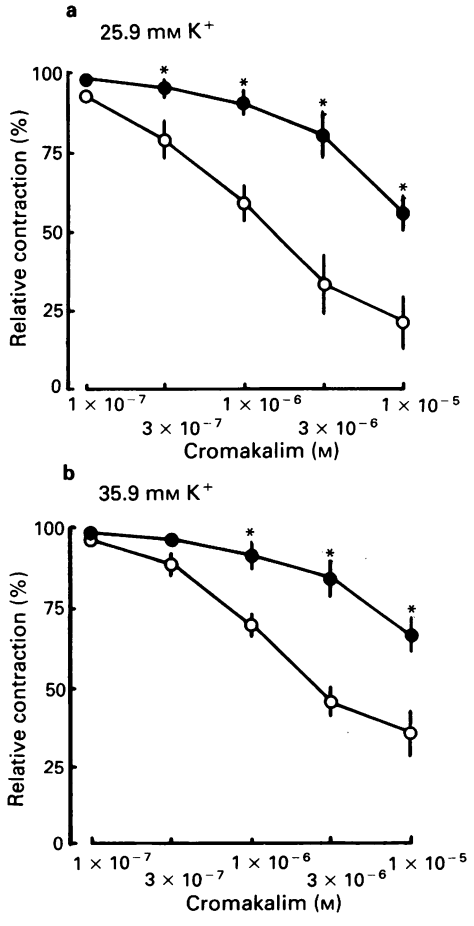

Figure 3 The effects of cromakalim on the high K^+-induced tonic contraction in the guinea-pig stomach in the absence (○) and presence (●) of glibenclamide 1×10^{-6} M. (a) Hypertonically added 20 mM KCl (total 25.9 mM K^+); (b) hypertonically added 30 mM KCl (total 35.9 mM K^+). On the ordinates, 100% = the magnitude of the tonic tension just before the addition of cromakalim. When used, glibenclamide was applied 10 min before the addition of K^+. Data represent the means with the s.e.mean of 8 (a) or 7 (b) experiments. Significantly different from cromakalim alone: $*P < 0.05$, paired t test.

Comparison of the effects of cromakalim on the mechanical (Figure 1) and electrical (Figure 5) activity of stomach antrum indicates that cromakalim ($1–3 \times 10^{-7}$ M) decreased the amplitude of spontaneous contractions without any membrane hyperpolarization. Since these responses were measured separately in different conditions (a Magnus bath and a superfusion bath), we considered the possible involvement of such differences in the observed effects of cromakalim. Experiments were therefore designed to study the effects of cromakalim on spontaneous contractions in the superfusion bath which was used for the microelectrode study. In these experiments, cromakalim at 1×10^{-7} M did not, but 3×10^{-7} M did inhibit the

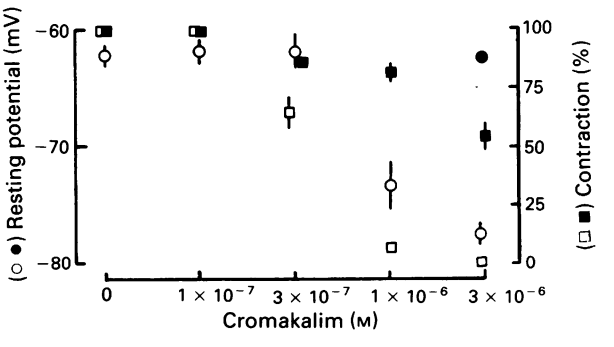

Figure 5 The effect of cromakalim on the resting membrane potential and the spontaneous contraction of the guinea-pig stomach. The contraction and the membrane potential were measured in the same superfusion bath but individually. (○, ●) Resting membrane potential; (□, ■) amplitude of spontaneous contractions; 100% = the amplitude of spontaneous contractions just before superfusion with cromakalim. Both parameters were measured at the peak effects of cromakalim; (■, ●) in the presence of glibenclamide 1×10^{-6} M. The effect of cromakalim on membrane potential in the presence of glibenclamide was tested only in a concentration of 3×10^{-6} M. Data represent the means with s.e.mean (vertical lines) of 6–8 experiments (resting potential) or 6 experiments (contraction).

spontaneous contractions ($pD_2 = 6.27 \pm 0.07$, $n = 6$); the inhibitory potency was slightly less than that observed in the Magnus bath (Figure 5).

Antagonistic effects of glibenclamide on actions of cromakalim

After a control response to cromakalim had been obtained in the absence or presence of stimulants, the muscles were rinsed with Krebs solution for 1 h, and then the response to cromakalim was again observed in the presence of 1×10^{-6} M glibenclamide. When control responses to cromakalim were observed twice with an interval of 1 h between responses, the second inhibitory effect was the same as the first one. Glibenclamide (1×10^{-6} M) alone did not modify the spontaneous contractions, those enhanced by ACh (1×10^{-6} M) or the tonic increase in tension induced by high $[K^+]_o$. However, this agent significantly attenuated the inhibitory actions of cromakalim on these contractions (Figures 1, 2, 3). A concentration above 3×10^{-6} M of cromakalim caused a considerable inhibition of spontaneous contractions even in the presence of glibenclamide (Figure 1) and the effects of cromakalim on spontaneous contractions were similarly resistant to glibenclamide in the superfusion bath (Figure 5).

The resting membrane potential and configurations of spontaneous electrical activities were not detectably affected by glibenclamide (1×10^{-6} M), but the actions of cromakalim on these electrical properties were completely prevented by glibenclamide (Figure 6).

Effects of charybdotoxin on the spontaneous contractions and on the action of cromakalim

Charybdotoxin enhanced the spontaneous contractions. The threshold concentration for this effect was 3×10^{-9} M and the maximum effect was attained at 1×10^{-7} M. With 3×10^{-8} M charybdotoxin, the spontaneous contractions were enhanced to $524 \pm 124\%$ ($n = 7$) with a small elevation of the basal tension (Figure 7a). Even in the presence of both 1×10^{-6} M atropine and 1×10^{-6} M tetrodotoxin, this increase in mechanical activity was observed, suggesting a direct action of the toxin on the gastric smooth muscle. Cromakalim added in the presence of charybdotoxin decreased the amplitude of charybdotoxin-enhanced spontaneous contractions in a dose-dependent manner (Figure 7b) with a pD_2 of 6.57 ± 0.05 ($n = 7$). This value was not significantly different from the pD_2 for the inhibition of the spontaneous contractions by cromakalim in control conditions.

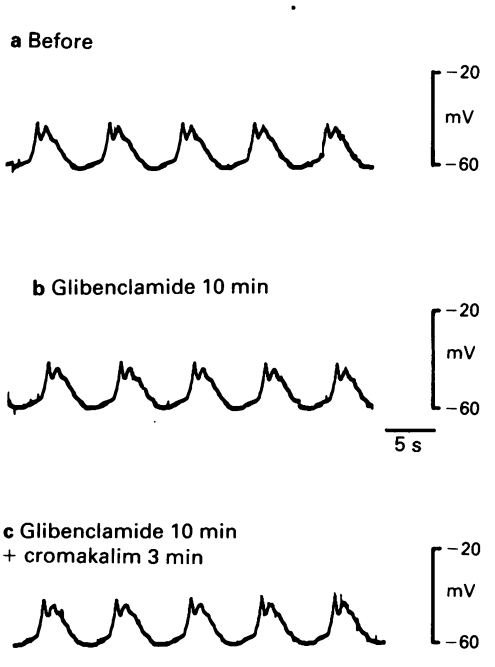

Figure 6 The antagonism of glibenclamide, 1×10^{-6} M, to the effect of cromakalim, 3×10^{-6} M, on the membrane potential of the guinea-pig stomach: (a) just before the addition of the drugs; (b) 10 min after superfusion with glibenclamide, which was just before superfusion with cromakalim; (c) 3 min after superfusion with cromakalim.

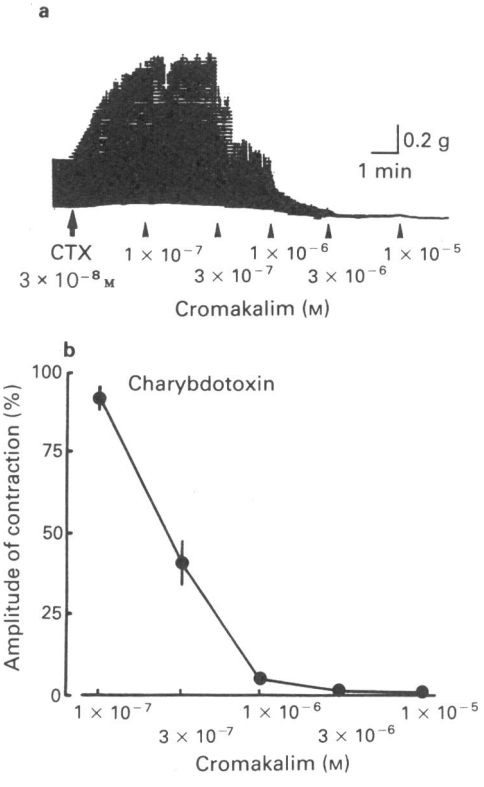

Figure 7 An example of the effect of cumulatively added cromakalim on the charybdotoxin (CTX)-enhanced spontaneous contractions of the guinea-pig stomach (a) and the summarized dose-effect relationship of cromakalim in the presence of charybdotoxin, 3×10^{-8} M (b). In (a) after the spontaneous contractions enhanced by charybdotoxin 3×10^{-8} M, reached the steady level, cromakalim was added cumulatively. In (b) 100% on the ordinate scale = the amplitude of enhanced spontaneous contractions just before the addition of cromakalim. Data represent the means with the s.e.mean (vertical lines) of 7 experiments.

Discussion

In this study cromakalim inhibited spontaneous contractions and those enhanced by ACh, with pD_2 values comparable to those previously observed in other smooth muscles. Cromakalim was less potent in inhibiting contractions produced in high $[K^+]_o$ than in normal $[K^+]_o$. This reduced effectiveness of cromakalim on KCl-induced contractions is in good agreement with other smooth muscle tissues (Allen *et al.*, 1986; Hamilton *et al.*, 1986; Hollingsworth *et al.*, 1987; Masuzawa *et al.*, 1990a,b) and is a first indication of the possible involvement of K^+ channel opening in the inhibitory actions of cromakalim.

In the guinea-pig stomach the threshold concentration of cromakalim required for inhibiting the spontaneous contractions was 1×10^{-7} M in the Magnus bath or 3×10^{-7} M in the superfusion bath, whereas that for the membrane hyperpolarization was 1×10^{-6} M. A typical feature of the cromakalim-induced inhibition of spontaneous contractions

without a change in the membrane potential can be seen at a concentration of 3×10^{-7} M, while higher concentrations of cromakalim inhibited both electrical and mechanical responses. Thus, the inhibitory actions of cromakalim on spontaneous contractions are not necessarily correlated with the membrane hyperpolarization or with inhibition of spontaneously generating electrical activities, as first observed in rat portal vein (Hamilton *et al.*, 1986).

Glibenclamide (1×10^{-6} M) antagonized the inhibitory effects of cromakalim on the spontaneous activities of the guinea-pig stomach. However, the actions of glibenclamide differed between electrical and mechanical responses, as the inhibition by 3×10^{-6} M cromakalim of the electrical activities was completely antagonized, while that of the mechanical activities was only partially blocked by glibenclamide. These results again suggest that the inhibitory actions of cromakalim on contractions are not solely due to membrane hyperpolarization. Some studies have claimed that cromakalim opens a Ca^{2+}-activated K^+ channel in smooth muscle cells of the guinea-pig mesenteric artery and rat portal vein (Nakao *et al.*, 1988; Hu *et al.*, 1990). However, the action of cromakalim on guinea-pig stomach was not affected by charybdotoxin, which is considered to block one type of Ca^{2+}-activated K^+ channel (Talvenheimo *et al.*, 1988; Strong *et al.*, 1989; Carl *et al.*, 1990a). If charybdotoxin is selective for such a channel, this result means that cromakalim does not activate the Ca^{2+}-activated K^+ channel sensitive to charybdotoxin in the guinea-pig stomach. Other studies have postulated that cromakalim (especially at concentrations $>1 \times 10^{-5}$ M) inhibits contractions of vascular smooth muscles and colonic smooth muscles partly through an inhibition of Ca^{2+} channels (Nakao *et al.*, 1988; Okabe *et al.*, 1990; Post *et al.*, 1991). Post *et al.* (1991) found that glibenclamide did not antagonize the Ca^{2+} channels blocking action of cromakalim in canine colonic smooth muscle cells. In the present study, cromakalim did not inhibit spike potentials and slow waves when glibenclamide was present. Therefore, the possible role of a Ca^{2+} channel in the inhibitory effects of cromakalim is unlikely in the guinea-pig stomach.

To explain the cromakalim-induced inhibition of spontaneous activity without hyperpolarization in the rat portal vein and uterus (Hamilton *et al.*, 1986; Hollingsworth *et al.*, 1987), these workers suggest that K^+ channels associated with the pacemaker activity in these tissues were more sensitive to this agent than those responsible for determining the resting membrane potential. This may not be the case in the guinea-pig stomach, because the frequency of the spontaneous slow waves was not changed by cromakalim at concentrations which inhibited spike generation. However, true slow waves are not generated either by the portal vein or the uterus, and further work is clearly required to establish the basis of cromakalim-induced inhibition in the absence of hyperpolarization.

Taking the above discussion into consideration, the lack of any change not only in the resting membrane potential but also in the slow waves and spikes during cromakalim-induced inhibition of contraction in the presence of glibenclamide suggests that a part of the inhibitory action of cromakalim on mechanical activity does not depend on the effects on ionic channels, or is related to the action on channels which cannot be detected by conventional microelectrode technique. The dissociation between electrical effects and mechanical effects of cromakalim was also observed by Tomita & Brading (1990), who showed that cromakalim suppressed the contraction of guinea-pig stomach in spite of the persistance of the slow wave. It was shown that cromakalim does not change the Ca^{2+}-sensitivity of smooth muscles (Allen *et al.*, 1986; Hollingsworth *et al.*, 1987). Therefore, the possible inhibition by cromakalim of an increase in $[Ca^{2+}]_i$ through potential-independent mechanisms, for example inhibition of intracellular Ca^{2+} refilling (Bray *et al.*, 1991), is suggested.

Charybdotoxin considerably potentiated spontaneous contractions. If this potentiation is assumed to be mediated by an action on a Ca^{2+}-activated K^+ channel, this would support the view that a Ca^{2+}-activated K^+ channel plays a role in the slow wave of gastric smooth muscle (Mitra & Morad, 1985; Carl *et al.*, 1990b). The inhibition of such a channel may prolong the open time of voltage-dependent Ca^{2+} channels and thereby augment Ca^{2+} entry. Microelectrode studies, which are now being undertaken, will clarify how this toxin modifies the resting membrane potential, the slow waves and spikes. On the other hand, glibenclamide did not affect spontaneous activity. Although there is disagreement about whether the K^+ channel sensitive to cromakalim and glibenclamide in smooth muscles can be identified as an ATP-sensitive K^+ channel (Beech & Bolton, 1989; Standen *et al.*, 1989; Fujii *et al.*, 1990; Hu *et al.*, 1990), the ineffectiveness of glibenclamide on spontaneous activity suggests that the K^+ channel sensitive to glibenclamide does not play a role under physiological conditions.

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(Received May 7, 1991
Revised October 16, 1991
Accepted October 21, 1991)