Dual Effects of Cyclopiazonic Acid on Excitation of Circular Smooth Muscle Isolated from the Guinea-pig Gastric Antrum

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Abstract

The effects of cyclopiazonic acid (CPA), a known Ca^{2+} -pump inhibitor at internal stores, were investigated on electrical responses of the membrane of smooth muscle cells in small segments (0.3–0.5 mm long) of circular smooth muscle isolated from the guinea-pig gastric antrum. In most preparations, the membrane was spontaneously active with the generation of unitary potentials and regenerative slow potentials. Low concentrations ($< 1 \mu\text{M}$) of CPA did not alter either the membrane potential or the amplitude and frequency of slow potentials. CPA at a concentration of $1 \mu\text{M}$ initially increased the frequency of slow potentials, but this was followed by a decrease in the frequency as a result of sustained exposure to CPA, with no alteration of either the membrane potential or the amplitude of slow potentials. Higher concentrations of CPA ($2\text{--}5 \mu\text{M}$) depolarized the membrane and decreased the amplitude and frequency of slow potentials. CPA at higher than $10 \mu\text{M}$ abolished slow potentials with depolarization of the membrane. Intracellular electrical responses recorded simultaneously from paired cells were synchronized, indicating electrical coupling of the cells. Depolarization of the membrane with current stimuli through one electrode evoked regenerative slow potentials superimposed on the electrotonic potentials. The evoked slow potential had a refractory period of about 7 s. CPA (up to $10 \mu\text{M}$) did not prevent the synchronization of paired cells. The refractory period for slow potentials was reduced by low concentrations of CPA ($< 1 \mu\text{M}$) and increased by higher concentrations of CPA ($2\text{--}10 \mu\text{M}$). These results suggest that lower concentrations of CPA produce excitatory actions on gastric smooth muscles due to a secondary effect of increased intracellular $[\text{Ca}^{2+}]$, while higher concentrations of CPA produce inhibitory actions as a result of reduced release of Ca^{2+} from depleted internal stores.

Key words: Slow potential, Depolarization, Refractory period, Calcium stores

Introduction

Spontaneous electrical activity of gastrointestinal smooth muscle cells as evidenced by slow waves and spike potentials has been considered to be myogenic in origin (Tomita, 1981). However, Thuneberg (1982) proposed that interstitial cells of Cajal (ICC), on the basis of their distribution in intestinal smooth muscle, were pacemaker cells for gastrointestinal motility. Maeda *et al.* (1992) reported that in mice, mutation of the *c-kit* gene induces serious disorders in gastrointestinal motility, with an associated inhibition of the development of interstitial cells (IC). These findings suggested that IC were central to the control of myogenic activity. Electrophysiological and immunohistochemical observations of gastrointestinal tissue have confirmed that the absence of IC occurs in parallel with the disappearance of slow waves in the intestine (Ward *et al.*, 1994; Huizinga *et al.*, 1995; Torihashi *et al.*, 1995). These studies have resulted in a reconsideration of the possible involvement of IC in gastrointestinal disorders (Sanders, 1996; Huizinga *et al.*, 1997; Sanders *et al.*, 1999). In the guinea-pig, rat and mouse, many types of IC are identified in the gastric wall, such as those distributed in myenteric layers (IC-MY) and those found within circular muscle bundles (IC-IM) (Komuro *et al.*, 1996; Burns *et al.*, 1997; Komuro *et al.*, 1999; Takayama *et al.*, 2002). The pacemaker cells for gastric activity may be the IC-MY which generate driving potentials with an initial fast spike followed by a plateau component of 10–15 s duration. These potentials are propagated to both the circular and longitudinal smooth muscle in an electrotonic manner through gap junctions (Dickens *et al.*, 1999). Slow waves generated in gastric smooth muscle of the guinea-pig consist of voltage-sensitive and voltage-insensitive components (Ohba *et al.*, 1975), or 1st and 2nd components (Tomita, 1981), and only the voltage-sensitive component is easily inhibited by caffeine (Dickens *et al.*, 1999). The driving potential generated in IC-MY is propagated to the circular muscle to form the voltage-insensitive component (or 1st component) of slow waves, and this potential then triggers a voltage-sensitive component (or 2nd component) (Dickens *et al.*, 1999). In the gastric muscle of *W/W^v* mutant mice lacking IC-IM, the caffeine-sensitive component of slow waves is absent (Dickens *et al.*, 2001), suggesting that this component is elicited by electrotonic spread of potentials generated in the IC-IM.

In isolated circular muscle of the guinea-pig antrum without attached IC-MY, regenerative slow potentials appear periodically (Suzuki and Hirst, 1999; Nose *et al.*, 2000; Fukuta *et al.*, 2002; Hirst *et al.*, 2002). These slow potentials are sensitive to caffeine and could be evoked by depolarization of the membrane, with a minimum latency of about 1 s (Suzuki and Hirst, 1999). When two depolarizing stimuli are applied to a segment of circular muscle from the guinea-pig gastric antrum, slow potentials are generated with a refractory period of about 5 s, which may be one of the important factors for the determination of the maximum frequency of slow waves (Nose *et al.*, 2000). The long latency for the generation of slow potentials is considered to be necessary for the production of unidentified messengers such as inositol trisphosphate (IP₃) in response to depolarization of the membrane (Suzuki *et al.*, 2000; Suzuki, 2000). The refractory period of slow potentials is also considered to be due to the time required for the resetting of the production of unidentified messengers. If this is the case, internal Ca²⁺ stores may be playing an important role in the generation of slow potentials, since IP₃ releases Ca²⁺ from internal stores

(Somlyo and Somlyo, 1994).

Cyclopiazonic acid (CPA) is a known Ca^{2+} -pump inhibitor at internal stores (Uyama *et al.*, 1992). Experiments were carried out to test the effects of CPA on slow potentials generated in isolated segments of circular smooth muscle of the guinea-pig gastric antrum. A small segment of smooth muscle which was about one-tenth shorter than the length constant of the tissue (equal to 2.2 mm, Osa & Kuriyama, 1970), was impaled by two intracellular electrodes, and electrical responses recorded simultaneously from two different cells, as described previously (Suzuki and Hirst, 1999). Cells were stimulated by current injection to one electrode, and the effects of CPA on the evoked electrical responses were tested with the other electrode. The results indicated that CPA has dual actions on the generation of slow potentials; low concentrations enhance and high concentrations inhibit the generation of slow potentials. Some of these experiments were reported briefly to the 43th Annual Meeting of the Japanese Smooth Muscle Society (Suzuki *et al.*, 2001).

Materials and Methods

Male albino guinea-pigs, weighing 250–300 gr, were anesthetized with fluoromethyl 2,2,2-trifluoro-1-(trifluoromethyl) ethyl ether (sevoflurane; Maruishi Pharm., Osaka, Japan) and decapitated. All animals were treated ethically in accord with the principles for the care and use of animals as approved by The Physiological Society of Japan. The stomach was excised, placed in Krebs solution and opened by cutting along the lesser curvature. The mucosal layers were removed by cutting with fine scissors, and smooth muscle tissue isolated from the antrum region. The circular tissue preparation (a single bundle 80–100 μm wide and 200–250 μm long) was prepared by mechanical removal of the longitudinal muscle layer with fine forceps. The preparation was pinned out on a Sylgard plate (silicone elastomer, Dow Corning Corporation, Midland, Michigan, U.S.A) at the bottom of the recording chamber (volume, approximately 0.5 ml), and superfused with warmed (35°C) Krebs solution at a constant flow rate of about 2 ml/min. The recording chamber was mounted on the stage of an inverted microscope (Nikon Diaphot 20, Tokyo, Japan). These methods were essentially the same as those reported by Suzuki and Hirst (1999).

Electrical responses of smooth muscle cells were recorded using conventional microelectrode methods. Glass capillary microelectrodes filled with 0.5 M KCl had a tip resistance of between 150 and 250 M Ω . Two microelectrodes were inserted into the same tissue, and electrical responses recorded simultaneously from two cells. Experiments were carried out when the signals recorded from the two electrodes were synchronized. Current pulses of 5–10 nA intensity and 1 s duration were applied to one electrode, and the electrotonic potentials produced were recorded by the second electrode. Membrane potential changes, recorded using a high input impedance amplifier (Axoclamp-2B, Axon Instruments, Inc., Foster City California, U.S.A), were displayed on a cathode-ray oscilloscope (SS-7602, Iwatsu, Osaka, Japan) and stored on a personal computer for later analysis.

The ionic composition of the Krebs solution was as follows (in mM): Na^+ 137.4; K^+ 5.9, Mg^{2+} 1.2, Ca^{2+} 2.5, HCO_3^- 15.5, H_2PO_4^- 1.2, Cl^- 134.0, glucose 15.5. The solutions were aerated with O_2

containing 5% CO₂, and the pH of the solution was 7.1–7.2.

Drugs used were caffeine, cyclopiazonic acid (CPA) and nifedipine (Sigma Chem., U.S.A.). Nifedipine and CPA were dissolved in dimethyl sulphoxide (DMSO), to make stock solutions, and they were added to Krebs solution to make the desired concentration. The volume ratios of the dilution of these chemicals were more than 1:1000. Caffeine was dissolved directly in Krebs solution to make a 1 mM solution. The dilution procedures did not alter the pH of the Krebs solution.

Values measured were expressed as the mean $\pm$ standard error of the mean (S.E.) or standard deviation of the mean (S.D.). Differences between values were tested using the unpaired Student *t*-test, and probabilities of less than 5% ($P < 0.05$) were considered to be significant.

Results

Effects of CPA on resting membrane potential and spontaneous electrical responses

Most of the cells examined demonstrated periodical generation of regenerative slow potentials, with a resting membrane potential of between -63.7 mV and -68.6 mV (mean, -66.2 ± 1.6 mV, $n=18$). A burst of spike potentials was often generated on top of each slow potential. The membrane potentials between slow potentials were not stable, and a random generation of transient small fluctuations (unitary potentials, Edwards *et al.*, 1999) was observed. Nifedipine ($1 \mu\text{M}$) abolished the spike potentials, with no interruption to the generation of slow potentials nor the unitary potentials. In the presence of nifedipine, slow potentials with amplitudes ranging between 25 mV and 45 mV (mean, 34 ± 3 mV, $n=18$) and duration (measured across the base of the potential) ranging between 5 s and 9 s (mean, 7.0 ± 0.5 s, $n=18$) were generated. These properties of the electrical responses were similar to those reported by Suzuki and Hirst (1999) and Fukuta *et al.* (2002).

Experiments were carried out in the presence of $1 \mu\text{M}$ nifedipine, to rule out a possible involvement of voltage-gated L-type Ca²⁺-channels in the actions of CPA. The effects of CPA on membrane potentials were investigated by exposing tissues to CPA-containing solution for over 15 min. Low concentrations of CPA ($< 1 \mu\text{M}$) did not alter the membrane potential, while high concentrations ($> 2 \mu\text{M}$) depolarized the membrane in a concentration-dependent manner (Fig. 1). It took more than 20 min for the CPA-induced depolarization to revert to the resting potential level following removal of CPA from the superfusate (data not shown).

Figure 2 shows the effects of four concentrations (0.5, 1, 3 and $10 \mu\text{M}$) of CPA on spontaneously generating slow potentials in antrum smooth muscle, in the presence of $1 \mu\text{M}$ nifedipine. CPA at a concentration of $0.5 \mu\text{M}$ (Fig. 2, B) did not alter the frequency of spontaneous slow potentials (control, $1.08 \pm 0.31 \text{ min}^{-1}$; in CPA, $1.03 \pm 0.23 \text{ min}^{-1}$; $n=8$; $P > 0.05$). CPA at a concentration of $1 \mu\text{M}$ (Fig. 2, D) increased the frequency of slow potentials (control, $1.49 \pm 0.23 \text{ min}^{-1}$; in CPA, $1.63 \pm 0.34 \text{ min}^{-1}$; $n=5$; $P < 0.05$) with no alteration of the resting membrane potential. The increased frequency of slow potentials by $1 \mu\text{M}$ CPA was a transient phenomenon (for up to 40 min). Continued exposure of tissues to this concentration of CPA for over 60 min resulted in a decrease in the frequency to levels similar to those before application

Fig. 1 Effects of CPA on membrane potentials. Membrane potentials were measured from circular smooth muscle cells in bundles from the guinea-pig gastric antrum in the absence (Control) and presence of CPA (0.3–10 μ M) for more than 30 min. Mean $\pm$ S.D. (n=6–15). p, significantly different from Control ($P<0.05$).

Fig. 2 Effects of CPA on spontaneous activity in gastric smooth muscle. Electrical responses were recorded from circular smooth muscle cells in bundles from the guinea-pig gastric antrum, in the absence (A, C, E and G) and presence of CPA (B, 0.5 μ M; D, 1 μ M; F, 3 μ M; H, 10 μ M). Each pair of responses was recorded from the same tissue.

of CPA (in CPA for 60–90 min, $1.43 \pm 0.25 \text{ min}^{-1}$; $n=5$; $P>0.05$). Increasing the concentration of CPA to $3 \mu\text{M}$ (Fig. 2, F) depolarized the membrane and reduced the frequency and amplitude of slow potentials (control, $1.39 \pm 0.40 \text{ min}^{-1}$; in CPA, $1.09 \pm 0.30 \text{ min}^{-1}$; $n=5$; $P<0.05$). Increasing the concentrations of CPA to $10 \mu\text{M}$ abolished slow potentials (Fig. 2, H). Thus, CPA has dual actions on slow potentials, excitatory actions at low concentrations and inhibitory actions at high concentrations.

Depolarization-evoked slow potentials

In more than 95% of the cell pairs examined in the circular muscle bundles isolated from the guinea-pig gastric antrum, electrical responses recorded simultaneously from the two cells were synchronized, indicating that they were electrically coupled. Application of current pulses (1–2 s duration) through one electrode produced electrotonic potentials in the other cell, which could be recorded by the second electrode. The electrotonic potentials thus produced could be recorded synchronously in all cell pairs. Current injection via one electrode produced a depolarizing potential in the second electrode, and the amplitude of the electrotonic depolarization increased with the amplitude of the injected current. When the amplitude of depolarization exceeded a certain level, the muscle cells produced regenerative slow potentials.

Attempts were made to stimulate the muscle cells with depolarizing pulses of supramaximal intensity ($> 5 \text{ nA}$) at various times after the cessation of a spontaneously generated slow potential. When pulses were applied within 1–2 s of the cessation of a spontaneous slow potential, only electrotonic potentials were produced (Fig. 3, A). Pulses applied 3–7 s after the cessation of a spontaneous slow potential evoked electrotonic potentials and subsequent small incomplete slow potentials, which looked like a cluster of unitary potentials (Fig. 3, B). Stimulation of the muscle cells 7–15 s after the spontaneous slow potentials elicited slow potentials with amplitudes similar to those generated spontaneously (Fig. 3, C). The relationship between the time of application of pulses after cessation of the spontaneous slow potentials and the peak amplitude of the evoked potentials (Fig. 3, D) indicated that there was a period of 1–2 s during which no response could be evoked by depolarizing pulses. During the 2–7 s period following cessation of slow potentials, depolarizing responses evoked by stimulating pulses exhibited an approximately linear increase. Stimulation of muscle cells more than 7 s afterwards produced regenerative slow potentials only. As a consequence, the relationship between the interval between cessation of a slow potential and delivery of the stimulus and the amplitude of the evoked response had a discontinuity around 7 s after cessation of the slow potentials. Thus, there was a refractory period of about 7 s for the generation of slow potentials.

In some preparations, the frequency of slow potentials decreased with time and finally disappeared. This usually occurred about 5–6 hours after the experiments commenced. In the absence of spontaneous slow potentials, slow potentials could still be evoked by stimulation of the muscle cells with depolarizing pulses. Experiments were carried out to compare the refractory period between spontaneous and evoked slow potentials. The refractory period of the evoked slow potential was measured by stimulating muscle cells with two pulses, one as a conditioning stimulus and the second as a test stimulus to elicit an evoked slow potential (see

Fig. 3 Slow potentials evoked by depolarizing pulses. During simultaneous recordings of membrane potentials from two cells in bundle of circular smooth muscle from the guinea-pig gastric antrum, current pulses with 3 nA intensity and 1.5 s duration were applied to one electrode, and the evoked responses were recorded from the second electrode. Current pulses were applied about 2 s (A), 4 s (B) and 8 s (C) after the cessation of a spontaneous slow potential. D, the relationship between the time of application of current pulses after cessation of slow potentials and the amplitude of potentials evoked by the depolarization. The sigmoidal curve in the figure is the curve of best fit of the data. Control, amplitude of slow potentials generated spontaneously before application of current pulses (mean $\pm$ S.D., $n=16$).

Fig. 4). The amplitudes of both the spontaneous and evoked slow potentials were identical, and the relationship between each pair of potentials was also similar (Fig. 4, C). These results indicate that there is a refractory period following slow potentials in gastric smooth muscle cells, irrespective of whether they are spontaneous or evoked, and during this period the generation of a succeeding slow potential is inhibited.

Modulation of evoked slow potentials by cyclopiazonic acid

Experiments were carried out to test the effects of low ($0.5 \mu\text{M}$), medium ($1 \mu\text{M}$) and high ($3 \mu\text{M}$) concentrations of CPA on electrical responses evoked by depolarizing stimuli in isolated circular smooth muscle of the guinea-pig antrum, all in the presence of nifedipine ($1 \mu\text{M}$). Figure 5 shows the effects of $3 \mu\text{M}$ CPA on electrical responses recorded from a pair of synchronized cells. This concentration of CPA depolarized the membrane, but did not alter the synchronization of the two cells. In all synchronized cell pairs examined, CPA (up to $10 \mu\text{M}$) did not prevent coordinated activity.

The effects of $0.5 \mu\text{M}$ CPA on evoked slow potentials are shown in Fig. 6. In the absence of CPA, a depolarizing pulse applied about 5 s after cessation of a spontaneous slow potential produced an electrotonic potential followed by several unitary potentials (Fig. 6, A). In the

Fig. 4 Refractory periods for spontaneous and evoked slow potentials. Smooth muscle cells were stimulated by depolarizing pulses (1.5 s duration, 2 nA intensity) after spontaneous slow potentials (A) or evoked slow potentials (B). In A and B, upper and lower traces indicate membrane potential and stimulating current, respectively. C, The relationship between the time for stimulation after cessation of slow potentials and amplitude of responses elicited by depolarizing pulses. Filled and open circles indicate responses in muscle cells with spontaneous and evoked slow potentials, respectively. The mean amplitudes ($\pm$ S.D.) of spontaneous slow potentials (filled circle) or evoked slow potentials (open circle) are shown to the left of the curves. These are not significantly different ($P>0.05$).

Fig. 5 Effects of CPA on synchronization of cells in a gastric smooth muscle bundle. Electrical responses were recorded from a pair of cells in a gastric circular smooth muscle bundle, in the absence (A and B) and presence of 3 μ M CPA (C and D). A–C and B–D were continuous recordings, with about 15 min interruptions.

Fig. 6 Effects of $0.5 \mu\text{M}$ CPA on slow potentials. Simultaneous recording of membrane potentials from two cells in a segment of circular smooth muscle tissue of the guinea-pig gastric antrum. The muscle cells were stimulated twice by current pulses of 1.5 s duration and 10 nA intensity with an interval of about 5 s, in the absence (A, Control) and presence of $0.5 \mu\text{M}$ CPA (B). A and B were recorded from the same pair of cells. C, the relationship between the time of application of the stimulating pulses after cessation of spontaneous slow potentials and the amplitude of responses evoked by the pulses, in the absence (filled circles) and presence of $0.5 \mu\text{M}$ CPA (open circles). The values shown in the left hand side (mean $\pm$ S.D.) are the amplitude of slow potentials generated spontaneously in the absence and presence of CPA. These are not significantly different ($P > 0.05$).

presence of $0.5 \mu\text{M}$ CPA, however, stimulation of the smooth muscle cells with a depolarizing pulse with the same intensity and duration elicited a slow potential (Fig. 6, B). In 5 preparations, the refractory period for generation of slow potentials was 6.4 ± 1.3 s in the absence of CPA, but was reduced to 5.2 ± 0.7 s ($P < 0.05$) in the presence of CPA, with no alteration of the membrane potentials (Fig. 1) and amplitude of evoked slow potentials (Fig. 6, C). Increasing the concentration of CPA to $1 \mu\text{M}$ did not produce any significant alteration in the resting membrane potential or in the amplitude of spontaneously generating slow potentials, as detailed above (Figs. 1 and 2). In the presence of $1 \mu\text{M}$ CPA for 20–40 min, the refractory period for generation of slow potentials was reduced to 4.8 ± 0.4 s ($n=3$, $P < 0.05$). However, the exposure of tissues to solutions containing $1 \mu\text{M}$ CPA for over 60 min resulted in an increase in the refractory period to 6.8 ± 1.3 s ($n=3$, $P < 0.05$) (Fig. 7). The effects of $3 \mu\text{M}$ CPA on slow potentials are shown in Fig. 8. Exposure of tissues to $3 \mu\text{M}$ CPA reduced the amplitude and frequency of slow potentials, and therefore the refractory period was measured on the evoked slow potentials (Fig. 8, B). In the presence of $3 \mu\text{M}$ CPA, the refractory period was increased to 9.4 ± 1.8 s ($n=5$, $P < 0.05$) (Fig. 8, C). In the presence of $10 \mu\text{M}$ CPA for over 30 min, no evoked slow potential was elicited by stimulation of smooth muscle cells with depolarizing pulses. These effects of CPA were reversible, with more than 30 min washing required for complete recovery (data not shown).

Fig. 7 Effects of 1 μ M CPA on slow potentials. Simultaneous recording of the membrane potentials from two cells in a segment of circular smooth muscle tissue of the guinea-pig gastric antrum. A 1 s current was applied to one electrode, and the evoked slow potentials recorded in the absence (filled circles) and presence of 1 μ M CPA for 22–35 min (open circles) and for 61–92 min (open triangles). The amplitudes of responses evoked in the second electrode were plotted as a function of the interval between the cessation of a spontaneous slow potential and the initiation of a current pulse. Mean amplitudes (mean $\pm$ S.D.) of slow potentials generated in the absence (filled circle) and presence of CPA for 22–35 min (open circle) and 61–92 min (open triangle) are shown at the left hand side of the graph. These are not significantly different from each other ($P > 0.05$).

Discussion

Spontaneous activity of gastrointestinal smooth muscle may be initiated by electrical signals conducted from IC-MY through gap junctions (Sanders, 1996; Huizinga *et al.*, 1997; Sanders *et al.*, 1999). In gastric smooth muscle of the guinea-pig, electrical activity of IC-MY appears prior to the generation of slow waves in circular smooth muscle, indicating that IC-MY are indeed driving electrical activity of gastric smooth muscle (Dickens *et al.*, 1999). However, isolated circular smooth muscle bundles of the guinea-pig gastric antrum still possesses spontaneous activity with generation of regenerative slow potentials, indicating that IC-MY are not the only pacemaker for gastrointestinal motility (Suzuki and Hirst, 1999; Suzuki, 2000). The slow potentials are generated in the presence of nifedipine, as in the case of slow waves (Suzuki and Hirst, 1999; Nose *et al.*, 2000; Fukuta *et al.*, 2002). These slow potentials differ from slow waves generated in intact circular smooth muscle tissues with attached longitudinal smooth muscle and IC-MY. The former are abolished by low concentrations (0.3–1 mM) of caffeine, while the latter are inhibited but never abolished by caffeine in concentrations up to 3 mM. The difference in the inhibitory potency of caffeine may be mainly related to the absence or presence of IC-MY in the tissue, since plateau-type driving potentials generated in IC-MY are inhibited

Fig. 8 Effects of 3 μM CPA on slow potentials. Slow potentials were elicited by depolarizing pulses (1.2 s duration, 3 nA intensity) at various times after cessation of spontaneous slow potentials (A). In the presence of 3 μM CPA, slow potentials were evoked by two stimuli (B). C, The relationship between the time for stimulation of muscle cells with depolarizing pulses and the amplitude of responses elicited by the pulses, in the absence (filled circles) and presence of 3 μM CPA (open circles). Mean amplitudes ($\pm$ S.D.) of spontaneous slow potentials (filled circle) or evoked slow potentials in the presence of CPA (open circle) are shown to the left of the curves. The mean amplitude of evoked slow potentials in the presence of CPA is significantly smaller than that for spontaneous slow potentials ($P < 0.05$).

only when high concentrations (>3 mM) of caffeine are applied (Dickens *et al.*, 1999). These data suggest that the slow potentials generated in circular smooth muscle without attached longitudinal muscle (and possibly IC-MY) are involved in the generation of slow waves in intact circular smooth muscle tissue. Slow waves recorded from the gastric antrum of the guinea-pig demonstrate both 1st and 2nd components with voltage-independent and dependent properties respectively (Ohba *et al.*, 1977). As low concentrations of caffeine inhibit only the 2nd component of slow waves (Dickens *et al.*, 1999), this component may be the potential generated in circular smooth muscle. Thus, the present experiments were carried out to test the effects of CPA on spontaneous and evoked electrical responses of circular smooth isolated from the guinea-pig gastric antrum muscle in the absence of functional IC-MY.

Smooth muscle cells are electrically coupled with other smooth muscle cells and also with IC-MY, and electrical responses of these cells are synchronized in the guinea-pig gastric antrum (Dickens *et al.*, 1999). In segments of the antral circular smooth muscle, electrical responses recorded from two cells were synchronized, indicating that gap junctions electrically coupled these cells. CPA (> 2 μM) depolarized the membrane, but did not alter the synchronization of the paired cells, suggesting that the intercellular electrical communication through gap junctions was not prevented by depletion of internal Ca^{2+} stores.

The present results indicate that slow potentials with a refractory period of about 7s may be elicited by depolarizing the membrane with current pulses. Thus, slow potentials are generated

in an all-or-none fashion, as in the case of nerve action potentials. In nerves, the refractory period for excitation is determined by the time required for the recovery of voltage-gated Na⁺-channels from the inactivation produced by membrane depolarization. In gastric muscle, depolarization of the membrane within the refractory period increased the generation of unitary potentials, in a time-dependent manner. The spectral analyses of the components of slow potentials indicate that they are formed as a sum of the irregularly generating unitary potentials (Edwards *et al.*, 1999), suggesting that the slow potential is produced by an instantaneous generation of unitary potentials. Depolarization of the membrane would accelerate the generation of unitary potentials, and when the amplitude of depolarization produced by the summed unitary potentials exceeded a certain level (threshold level), a slow potential may be generated. The unitary potentials are formed by an increased conductance of Ca²⁺-activated Cl⁻-channels (Hirst *et al.*, 2002). If this is the case, the evoked slow potentials may be produced as the result of an instantaneous increase in concentration of intracellular Ca²⁺ ([Ca²⁺]_i). It is of interest to note that slow waves are absent from the gastric muscle of mutant mice which lack IP₃ receptors (Suzuki *et al.*, 2000). Also, in circular smooth muscle bundles isolated from the guinea-pig gastric antrum, slow potentials are generated by depolarizing the membrane, with a minimum latency of about 1s (Suzuki and Hirst, 1999). This period of time is comparable to the delay required for the production of IP₃ in smooth muscle in response to stimulation with agonists (Somlyo and Somlyo, 1994). 2-Aminoethoxydiphenyl borate (2-APB), an inhibitor of the IP₃ receptor-mediated release of Ca²⁺ from internal stores (Maruyama *et al.*, 1992), abolishes slow waves (Hirst and Edwards, 2001) and also slow potentials (Fukuta *et al.*, 2002) in guinea-pig gastric smooth muscle. In murine small intestine, the inhibition of spontaneous activity by xestospongine C is mediated by blocking IP₃ receptors (Malysz *et al.*, 2001). These results suggest that the production of IP₃ may be one of the essential steps for the generation of slow potentials during depolarization, and the release of Ca²⁺ from internal stores by IP₃ would facilitate the generation of unitary potentials, possibly by activation of Ca²⁺-activated Cl⁻ channels (Hirst *et al.*, 2002). The increased production of receptor-mediated IP₃ during depolarization of the membrane (Ganitkevich and Isenberg, 1993; Mason and Mahaut-Smith, 2001) supports this suggestion.

There are a number of possible steps in the cellular mechanisms involved in the generation of slow potentials in the circular smooth muscle of the guinea-pig gastric antrum which have been presented by Fukuta *et al.* (2002) as follows. (1) Mitochondrial metabolic activity increases the production of protons, and extrusion of these protons from the mitochondria results in hyperpolarization of the mitochondrial inner membrane (Duchen, 1999). (2) Potentials produced across the mitochondrial membrane facilitate the influx of Ca²⁺ into the mitochondria from the cytosol. (3) Influx of Ca²⁺ will activate mitochondrial activity and consume mitochondrial ATP. (4) Reduction in mitochondrial ATP will activate ATP-sensitive K⁺-channels distributed on the mitochondrial inner membrane and depolarizes this membrane, which in turn forces an extrusion of Ca²⁺ from mitochondria. (5) Ca²⁺ released from mitochondria elevates production of IP₃ through activation of Ca²⁺-sensitive enzymes such as phospholipase C. (6) The elevated IP₃ causes the release of Ca²⁺ from the IP₃-sensitive internal stores, and (7) the elevation of [Ca²⁺]_i activates a set of ion channels at the plasma membrane to

produce membrane potential changes. These mechanisms are similar to those found in cultured IC-MY obtained from mouse small intestine (Ward *et al.*, 2001). The difference between cultured IC-MY and antral circular smooth muscle appears to be the site of initiation of the activity. In IC-MY, the release of Ca^{2+} from the internal store through IP_3 receptor activation starts a train of responses including Ca^{2+} handling in mitochondria (Ward *et al.*, 2001), while in antral circular smooth muscle, mitochondrial Ca^{2+} handling is the initial step of the spontaneous activity (Fukuta *et al.*, 2002). In gastric smooth muscle, 10 μM CPA depolarizes the membrane and increases $[\text{Ca}^{2+}]_i$ in the presence of nifedipine (Fukuta *et al.*, 2002). The membrane depolarization produced by this concentration of CPA was also confirmed in the present experiments. Depolarization of the membrane increases the frequency of slow potentials (Nose *et al.*, 2000), with an associated increase in $[\text{Ca}^{2+}]_i$ (Fukuta *et al.*, 2002). Reduction of $[\text{Ca}^{2+}]_i$ by BAPTA prevents the generation of slow potentials (Suzuki and Hirst, 1999; Hirst and Edwards, 2001; Fukuta *et al.*, 2002). These results suggest that an elevation of $[\text{Ca}^{2+}]_i$ is one of the factors to initiate slow potentials. Taken together, the inhibition of slow potentials by high concentrations of CPA ($> 2 \mu\text{M}$) may be caused by depletion of the internal calcium stores. However, it remains unclear how low concentrations of CPA increase the frequency of slow potentials. It is speculated that low concentrations of CPA cause a slight increase in $[\text{Ca}^{2+}]_i$ due to a partial inhibition of the Ca^{2+} -pump at the internal stores, however the internal stores could still be capable of releasing Ca^{2+} from the stores rhythmically in response to signals from mitochondria. As a consequence, elevation of $[\text{Ca}^{2+}]_i$ may accelerate generation of slow potentials.

The refractory period was similar for both spontaneous and evoked slow potentials, suggesting that these potentials are produced by similar mechanisms. Low concentrations of caffeine ($< 1 \text{ mM}$) equally inhibit both types of slow potentials (Suzuki and Hirst, 1999), which supports this suggestion. However, it remains unclear how the refractory period for generation of slow potentials is formed in the pacemaker cells. In gastric smooth muscle, the refractory period increases in the presence of high concentrations of thapsigargin and is decreased by acetylcholine (Suzuki *et al.*, 2001). In considering the role of IP_3 for the generation of slow potentials, the refractory period may be related to the time required for the supply of IP_3 , or alternatively the time required for filling the internal calcium stores. Thapsigargin depletes internal Ca^{2+} stores and acetylcholine increases the production of IP_3 through activation of muscarinic receptors (Somlyo and Somlyo, 1994). As CPA has actions similar to thapsigargin (Uyama *et al.*, 1992), the increase in the refractory period by high concentrations of CPA may be mainly due to the inhibition of Ca^{2+} release from the internal stores as a result of depletion of internal Ca^{2+} stores. The reduction of the refractory period that appeared in the presence of low concentrations of CPA, however, is not explained directly by the actions of CPA. The frequency of spontaneous slow potentials increases in the presence of low concentrations of CPA. The frequency of spontaneously generating slow potentials is a function of $[\text{Ca}^{2+}]_i$, as an increase in $[\text{Ca}^{2+}]_i$ accelerates the frequency (Fukuta *et al.*, 2002). Low concentrations of CPA will produce a partial inhibition of the Ca^{2+} -pump at the internal membrane, and this would result in a small increase in $[\text{Ca}^{2+}]_i$. Thus, it is speculated that the reduction of the refractory period for slow potentials may be secondary to the increase in $[\text{Ca}^{2+}]_i$ due to partial inhibition of Ca^{2+} -uptake into

internal stores. In the presence of 1 μM CPA, the inhibitory actions of CPA on the Ca^{2+} -pump may be nearly balanced by the extrusion of Ca^{2+} from the cell through unidentified pathways, and as a consequence $[\text{Ca}^{2+}]_i$ in pacemaker cells may be initially increased. Sustained exposure to CPA results in a continual depletion of Ca^{2+} from the internal stores. As the membrane potentials are not altered by low concentrations of CPA ($< 1 \mu\text{M}$), the level of $[\text{Ca}^{2+}]_i$ would be below threshold for the activation of Ca^{2+} -sensitive ion channels at the plasma membrane.

It is concluded that in gastric smooth muscle, low concentrations of CPA have excitatory actions, possibly due to an elevation of $[\text{Ca}^{2+}]_i$ as a secondary result of a partial inhibition of the Ca^{2+} -pump at the internal Ca^{2+} stores, while high concentrations of CPA have inhibitory actions mainly due to depletion of the internal Ca^{2+} stores.

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