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The properties of ryanodine-sensitive Ca^{2+} release in mouse gastric smooth muscle cells

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1 Under voltage-clamped conditions, gastric smooth muscle cells of BALB/c mice developed spontaneous (STOCs) and caffeine- (I_{CAF}) and carbachol-induced (I_{CCh}) transient outward currents.

2 In fura-2 microscopic measurements of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), caffeine and carbachol (CCh) provoked similar transient $[Ca^{2+}]_i$ elevations.

3 Both I_{CCh} and CCh-induced $[Ca^{2+}]_i$ elevation of single smooth muscle cells occurred in an 'all-or-nothing' fashion in contrast to the reproducible caffeine responses.

4 On the basis of the suppression of STOCs and I_{CAF} by nicardipine, tetraethylammonium and iberiotoxin, but not by charybdotoxin nor apamin, it was suggested that both currents were generated by large conductance type Ca^{2+}-activated K^+ channels.

5 In measurements of isometric tension, caffeine produced relaxation of gastric smooth muscle strips in a concentration-dependent manner (0.1–3 mM). The concentration-dependent relaxation with caffeine was mimicked by dibutyryl cyclic AMP which produced potentiation of contraction triggered by 50 mM KCl.

6 At caffeine concentrations >3 mM, a transient contraction followed by relaxation was provoked as the quasi maximal response to caffeine. In the quasi maximal response, caffeine acted as a potent relaxant in smooth muscle strips precontracted with 50 mM KCl or 3 μM CCh.

7 The relaxation with caffeine was significantly accelerated in those strips precontracted with KCl or CCh. All these results suggest that ryanodine-sensitive Ca^{2+} release, which is triggered by caffeine, is an important modifier of Ca^{2+} homeostasis in the cytoplasm and the contractility of gastric smooth muscle cells of mice.

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Abbreviations: BK-type channel, Ca^{2+}-activated K^+ channel with large conductance; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; $[Ca^{2+}]_o$, extracellular Ca^{2+} concentration; CaMKII, Ca^{2+}/calmodulin-dependent protein kinase II; cyclic AMP, adenosine 3′,5′-cyclic monophosphate; CCh, carbachol; dB-cAMP, dibutyryl adenosine 3′,5′-cyclic monophosphate; fura-2/AM, acetoxymethyl ester of fura-2; HEPES, *N*-2-hydroxy-ethylpiperazine-*N*′-2-ethane-sulphonic acid; IbTx, iberiotoxin; I_{CAF}, caffeine-induced transient outward currents; I_{CCh}, carbachol-induced transient outward currents; $InsP_3$, inositol 1,4,5-trisphosphate; *I-V*, current-voltage; STOCs, spontaneous transient outward currents; TEA, tetraethylammonium; τ_C, time constant for the contraction; τ_R, time constant for the relaxation; V_H, holding voltage

Introduction

The ryanodine receptor is a type of Ca^{2+}-releasing channel on the sarcoplasmic or endoplasmic reticulum, and plays an important role in Ca^{2+} homeostasis in the cytoplasm of neuronal (Peng, 1996; Smith & Cunnane, 1996), muscle (Yamazawa *et al*., 1997; Sham *et al*., 1998) and other cell types (Sundaresan *et al*., 1997; Sei *et al*., 1999).

Analysis of the DNA which encodes ryanodine receptors has revealed that at least three subtypes are present in this receptor family, and that the composition of these subtypes varies depending upon the tissue involved (Takeshima, 1993; Takeshima *et al*., 1998; Sonnleitner *et al*., 1998). In skeletal muscle, type I (skeletal muscle type) ryanodine receptors, together with dihydropyridine binding proteins, serve for voltage-induced Ca^{2+} release that triggers the key step of excitation-contraction coupling (Meissner, 1994; Nakai *et al*., 1996; Yamazawa *et al*., 1996). The role of ryanodine receptors in cardiac muscle is mediation of Ca^{2+}-induced Ca^{2+} release, and is fulfilled by type II (cardiac muscle type) ryanodine receptors (Nakai *et al*., 1990; Imagawa *et al*., 1992; Stern *et al*., 1999). In smooth muscle cells, cardiac type II ryanodine receptors are identified as the major population (Imagawa *et al*., 1992).

Despite the comparatively large population of cardiac type ryanodine receptors in smooth muscle cells, little is known about the role of ryanodine receptors in smooth muscle function, compared to inositol 1,4,5-trisphosphate ($InsP_3$)-dependent mechanisms (Somlyo *et al*., 1985; Iino & Endo, 1992). A reason is that ryanodine receptor-mediated smooth muscle functions, which are generally measured as responsiveness to caffeine, vary, depending upon the type of smooth

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muscle tissue. In rat aortic smooth muscle strips, caffeine is known to elicit contraction (Watanabe *et al*., 1992), while in bladder smooth muscle strips of BALB/c mice, caffeine acts as a relaxant (Sugita *et al*., 1998).

Since most of the studies on the roles of cytoplasmic Ca^{2+} release in the functions of smooth muscle tissues have been conducted with animals other than mice, probably because of the size of specimens, little is known about the genetic background for the functions of ryanodine receptors in the smooth muscle tissues. To obtain information on the genetic background for the functions of ryanodine-sensitive Ca^{2+} release in smooth muscle tissues, it is becoming important to work with mouse tissues since recent gene-targeting studies have made it possible to obtain so called 'knock out' mice that lack genes encoding specific proteins. Ryanodine receptor-deficient mice have been established with this technique (Takeshima *et al*., 1994; 1996).

In the previous study with standard BALB/c mice, we found that caffeine-induced Ca^{2+} release and carbachol (CCh)-induced Ca^{2+} release in bladder smooth muscle cells differed in their dependence upon extracellular Ca^{2+}, and that both caffeine-sensitive and CCh-sensitive Ca^{2+} storage seemed to partially overlap (Sugita *et al*., 1998).

In the present study, to clarify how ryanodine-sensitive Ca^{2+} release occurs and contributes to the function of mouse gastric smooth muscle as a representative specimen of gastrointestinal smooth muscle tissues, we analysed the Ca^{2+}-activated K^+ currents, $[Ca^{2+}]_i$ elevation, and muscular contraction in response to caffeine and other contractile stimulants.

Methods

Cell preparations

Single smooth muscle cells from mouse stomach were freshly dissociated as described previously (Sugita *et al*., 1998) with some modifications. Briefly, BALB/c mice at day 10–15 post-partum were decapitated under ether anaesthesia. The abdomen was opened and the entire stomach removed. The gastric corpus was dissected into small pieces (about 2×2 mm). The fragments were incubated for 25–30 min in oxygenated, Ca^{2+}-free external solution containing 2 mg ml^{-1} collagenase type II (Sigma, U.S.A.), 1 mg ml^{-1} papain (Wako, Japan), 5 mg ml^{-1} bovine serum albumin (Sigma, U.S.A.), 1 mg ml^{-1} trypsin inhibitor (Sigma, U.S.A.) and 1 mM dithiothreitol (Sigma, U.S.A.), at 31°C. After the enzyme treatment, the blocks of smooth muscle were rinsed with an external solution containing 0.8 mM $CaCl_2$ and triturated with a pasteur pipette in the normal external solution containing 2 mM $CaCl_2$ for dissociation of single smooth muscle cells. The dissociated gastric smooth muscle cells were placed on poly-L-lysine-coated coverslips in the centre of 35-mm plastic dishes (Meridian, U.S.A.) containing 2 ml normal external solution. After 1 h, the dissociated cells became available for patch-clamp experiments and measurement of $[Ca^{2+}]_i$, because they were strongly attached to the coverslips. The freshly dissociated smooth muscle cells were used the same day. All experiments were carried out according to the Guidelines for Animal Experiments at Kumamoto University School of Medicine.

Recordings of membrane currents

The whole-cell recordings of membrane currents were performed with the nystatin-perforated patch-clamp technique (Horn & Marty, 1988), under voltage clamp conditions at room temperature (22–25°C). Recording micropipettes made of Pyrex capillary tubes with 1.5 mm outer diameters (Narishige G1.5, Japan) were fabricated in two stages by use of a vertical puller (Narishige PB-7, Japan). The recording pipettes filled with the pipette solution containing nystatin at 0.1–0.3 mg ml^{-1} gave pipette resistance of 2–3 MΩ when measured in the normal external solution. This gave a pipette resistance of 2–3 MΩ in the normal external solution and the series resistance of 5–8 MΩ was not compensated. The whole cell ionic currents were low-pass filtered at 1 KHz and sampled at 3 KHz under voltage-clamp conditions through a patch/whole cell-clamp amplifier (Nihon Kohden CEZ2400, Japan). Membrane currents and membrane voltage were monitored on a digital storage oscilloscope (5103N, Tektronix, U.S.A.) and a pen recorder (FBR-251A, Shimazu-seisakusho, Japan), and stored on a digital recorder (Instrutech corp. VR-10B, U.S.A.) for subsequent analysis. The employed holding voltage was −20 mV unless otherwise specified. In some experiments, a set of sequential voltage-ramp stimulations from −70 to +40 mV over a period of 2 s were applied by a function generator (Kikusui 459AL, Japan). The amplitude of membrane currents was measured from the zero current level that was obtained by application of 10 mM tetraethylammonium (TEA). When the frequency of spontaneous currents was comparatively low, the baseline current was easily determined without application of TEA and was not affected by TEA.

Fura-2 measurements of $[Ca^{2+}]_i$

For measurements of $[Ca^{2+}]_i$, single gastric smooth muscle cells were prepared in the same way as for the electrophysiological experiments, and fura-2 microscopic measurements were performed with an Argus50/CA system (Hamamatsu, Japan). Briefly, single gastric smooth muscle cells fixed on a poly-L-lysine-coated glass coverslip in the centre of 35-mm plastic dishes (Matsunami Glass, Japan) were incubated in the normal external solution containing 5 μM acetoxymethyl ester of fura-2 (fura-2/AM) for 20 min at 37°C in a dark room. After loading with the fluorescent dye, the gastric smooth muscle cells were rinsed with normal external solution to remove the residual dye outside the cell, and were then equilibrated for 30 min at room temperature. The fura-2-loaded gastric smooth muscle cells were illuminated by alternately exciting them at 340 and 380 nm with a xenon lamp. Fluorescent images of the gastric smooth muscle cells seen through a microscope (Nikon, Diaphot-Tmd, Japan) were sampled at 0.2 Hz and were stored in an image processor (Hamamatsu, Argus-50, Japan) by means of a 510 nm bandpass filter and SIT camera (Hamamatsu, C2400, Japan). The ratio of the fluorescence intensity at 340 nm excitation (F340) to that at 380 nm excitation (F380) was monitored and computer processed (Hamamatsu, U4469, Japan). We calculated $[Ca^{2+}]_i$ from the fura-2 fluorescence ratio (R) using the following equation (Grynkiewicz *et al*., 1985):

$$[Ca^{2+}]_i = K_d(R - R_{min})/(R_{max} - R) \quad (1)$$

where K_D is the dissociation constant for fura-2 (224 nM, Grynkiewicz *et al*., 1985). R_{max} and R_{min} were determined by the addition of 5 mM ionomycin in the normal external solution, and in Ca^{2+}-free external solution containing 2 mM EGTA.

In both electrophysiological experiments and measurements of $[Ca^{2+}]_i$, drugs were rapidly applied by use of a multi-barrelled pipette (Carbone & Lux, 1987) or Y-tube technique (Murase *et al*., 1990). All experiments were performed at 22–25°C.

Measurements of isometric tension of gastric smooth muscle strips

Pieces of gastric corpus (3–4 mm length) were dissected from animals aged 10–15 days post-partum, and luminal contents were rinsed out with the same normal external solution as used in the electrophysiological experiments and measurements of $[Ca^{2+}]_i$. The strips were anchored to organ bath hooks and suspended in a classical organ bath set-up for isometric measurements (5 mN resting tension was applied). The organ baths were filled with the normal external solution kept at 37 ± 0.5°C and gassed with 95% O_2 and 5% CO_2. The strips were then equilibrated for at least 30 min before the experiments.

Experimental solutions and drugs

The normal external solution contained (in mM): NaCl 150, KCl 5, $MgCl_2$ 1, $CaCl_2$ 2, *N*-2-hydroxy-ethylpiperazine-*N*′-2-ethane-sulphonic acid (HEPES) 10 and glucose 10. The pipette solution contained (in mM): KCl 150 and HEPES 10. The pH of the external and pipette solutions was adjusted to 7.4 with NaOH (1 N) and 7.2 with KOH (1 N), respectively.

Substances used for the experiments were nystatin (Nakarai Chemical, Japan), fura-2/AM (Dojindo, Japan), ionomycin (Sigma, U.S.A.), caffeine (Sigma, U.S.A.), carbachol (Sigma, U.S.A.), nicardipine (Sigma, U.S.A.), tetraethylammonium (Wako, Japan), iberiotoxin (Peptide Institute Inc., Japan), charybdotoxin (Peptide Institute Inc., Japan), apamin (Peptide Institute Inc., Japan), ryanodine (Wako, Japan), and dibutyryl cyclic AMP (Wako, Japan).

Experimental data are given as mean ± s.e.mean, and the statistical significance was estimated by Student's unpaired *t*-test. *P* values of less than 0.05 were considered to be statistically significant.

Results

Caffeine-induced outward current and spontaneous outward currents

The resting membrane potential of gastric smooth muscle cells was -51.2 ± 3.1 mV ($n=7$) under current-clamp conditions and the resting $[Ca^{2+}]_i$ was 214.3 ± 6.1 nM ($n=22$) when calculated from the fura-2 fluorescence ratio. Under voltage-clamped conditions at potentials positive to −50 mV with the nystatin-perforated patch-clamp technique, most freshly dissociated gastric smooth muscle cells ($n=83/88$) developed spontaneous transient outward currents (STOCs) and responded to caffeine, generating a transient outward current (I_{CAF}) which was followed by an inward current with the cessation of STOCs. The frequency of STOCs was 21 ± 3.3 min^{-1} and their amplitude was 5.69 ± 0.62 pA at a holding voltage (V_H) of −20 mV ($n=11$).

Figure 1a shows representative examples of STOCs and I_{CAF} at −20 mV. The left and right panels in Figure 1a show the same current recording with different time scales. The open and closed triangles indicate currents evoked by voltage ramps (see Methods) before and during application of caffeine respectively (see Figure 1a, right panel). As shown by the dotted lines, the zero current levels for STOCs and I_{CAF} were obtained by application of 10 mM TEA. When the frequency of STOCs was comparatively low, the baseline current was easily determined without application of TEA and was not affected by TEA. The outward component of the membrane current to the voltage ramp stimulation decreased in amplitude to $70 \pm 1.5\%$ ($n=5$) of the control during the application of caffeine but not the inward component ($n=5$). This suggests that the membrane conductance was reduced just after the discharge of I_{CAF}. The baseline current following I_{CAF} was identical to the zero current level (Figure 2a). This was confirmed in all gastric smooth muscle cells tested ($n=11$).

In various types of smooth muscle cells, spontaneous and caffeine-evoked outward currents are known to contain a large component of Ca^{2+}-activated K^+ current (Benham & Bolton, 1986; Knot *et al*., 1998; Perez *et al*., 1999). Figure 1b shows STOCs and I_{CAF} at various V_H. Both outward currents were detected at V_H positive to −50 mV and were increased in amplitude on depolarization of V_H. No inward current was detected over the range of V_H tested, consistent with the results obtained with the voltage-ramp method as shown in Figure 1a. The amplitude of STOCs (closed circle) was measured by averaging the current recordings sampled for 1 min at each V_H (Figure 1b,c). The maximal amplitude for I_{CAF} (open circle) was measured at each V_H and plotted against V_H. The current-voltage (*I-V*) relationships for STOCs and I_{CAF} were obtained from 11 gastric smooth muscle cells as shown in Figure 1c. The *I-V* relationship for both currents revealed outward rectification, similar to that described for Ca^{2+}-activated K^+ currents of rabbit intestinal smooth muscle cells (Benham & Bolton, 1986), bovine ciliary muscle cells (Fujii *et al*., 1997) and mouse bladder smooth muscle cells (Sugita *et al*., 1998).

Pharmacological properties of I_{CAF} and STOCs

In vascular (Benham & Bolton, 1986) and intestinal smooth muscle cells (Bolton & Lim, 1989), both STOCs and I_{CAF} have been reported to be TEA-sensitive Ca^{2+}-activated K^+ currents. We studied the effects of blockers of Ca^{2+}-activated K^+ channels, including TEA, iberiotoxin (IbTx) (Galvez *et al*., 1990) and apamin, on both outward currents in the mouse gastric smooth muscle cells. Figure 2a shows representative effects of TEA and IbTx on STOCs and I_{CAF}. The TEA block of the currents was observed at 0.3 mM TEA and the extent of block increased in a TEA concentration-dependent manner (data not shown). Both currents were completely abolished by 10 mM TEA in all gastric smooth muscle cells tested ($n=7$). Both STOCs and I_{CAF} were also suppressed by IbTx at 0.1 μM ($n=5$) but were completely

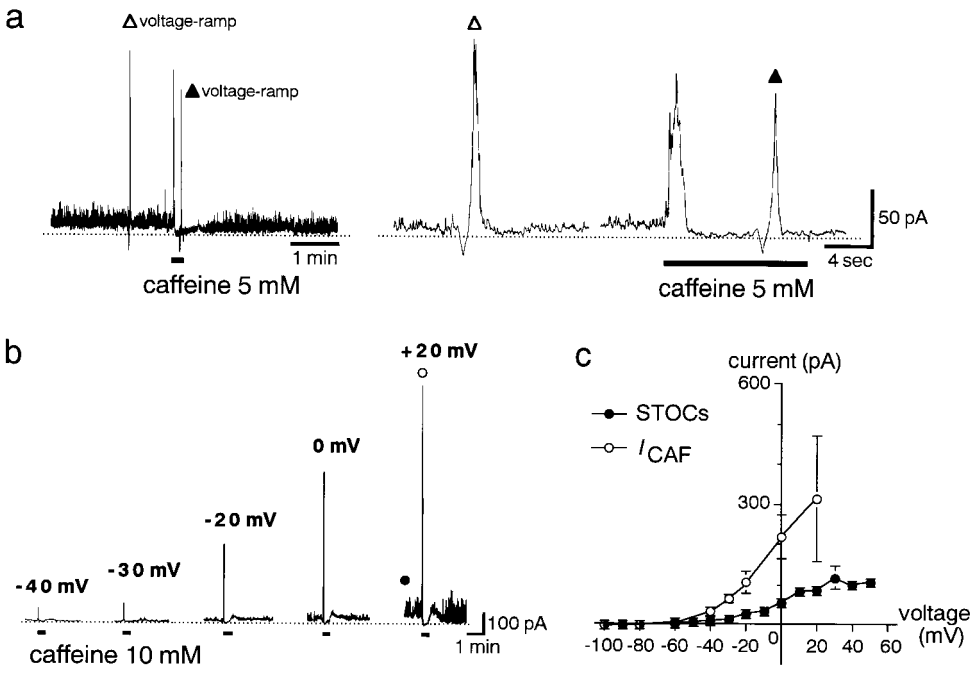

Figure 1 I_{CAF} and STOCs of mouse gastric smooth muscle cells. (a) I_{CAF} and current responses to a voltage-ramp stimulation to +40 mV and −70 mV from a V_H of −20 mV in the absence (open triangle) and in the presence (closed triangle) of caffeine. The same current recording is shown at different time scales. (b) STOCs and I_{CAF} at different V_H. Horizontal closed bars indicate application of caffeine. Dotted lines indicate the zero current level. The zero current level was obtained by application of 10 mM TEA. When the frequency of STOCs was comparatively low, the baseline current was easily determined without application of TEA and was not affected by TEA. (c) Instantaneous *I-V* relationships of STOCs and I_{CAF}. Abscissa: V_H, ordinate: amplitude of STOCs and I_{CAF}. Symbols and error bars indicate the mean±s.e.mean ($n=11$).

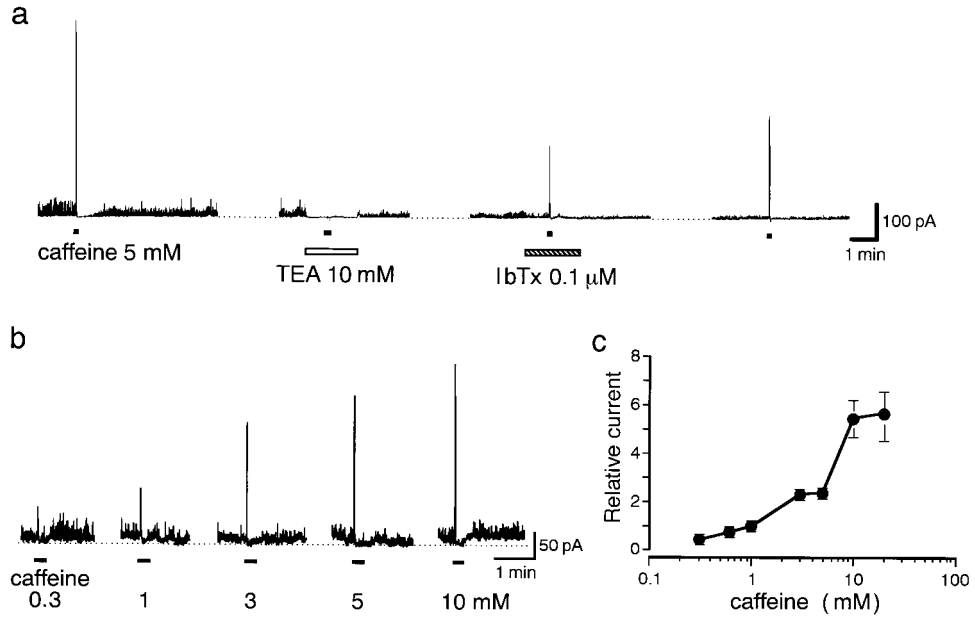

Figure 2 Pharmacological properties of I_{CAF} and STOCs. (a) Effects of TEA and IbTx on STOCs and I_{CAF} at a V_H of −20 mV. (b) I_{CAF} evoked at various concentrations of caffeine at −20 mV. Horizontal closed bars indicate application of caffeine, while open and striped bars indicate application of the K^+ channel blockers, TEA and IbTx. Dotted lines indicate the zero current level. (c) Caffeine concentration-I_{CAF} relationship. Abscissa: concentrations of caffeine, ordinate: relative I_{CAF} which was normalized to that evoked at 1 mM caffeine. Symbols and error bars indicate the mean±s.e.mean ($n=11$).

resistant to charybdotoxin up to 1 μM ($n=5$, data not shown) in all gastric smooth muscle cells tested. The effects of IbTx were partially reversible at 0.1 μM as shown in Figure 2a, while irreversible at >1 μM (data not shown). The EC_{50} values for TEA and IbTx to suppress I_{CAF} were 2.5 mM and 45 nM, respectively, when I_{CAF} was evoked by 5 mM caffeine.

The EC_{50} values for the K^+ channel blockers on STOCs have not yet been determined. Neither STOCs nor I_{CAF} were suppressed by apamin, up to 1 μM in all gastric smooth muscle cells tested ($n=5$, data not shown). These results suggest that TEA- and IbTx-sensitive but charybdotoxin- and apamin-resistant Ca^{2+}-activated K^+ channels are involved in each current.

Figure 2b shows I_{CAF} evoked at various concentrations of caffeine at a V_H of -20 mV. This V_H allowed reproducible I_{CAF} when evoked repeatedly at application intervals of >5 min. Caffeine was applied for long enough (10–15 s) to observe activation and decay of the current. To obtain the concentration-response relation for I_{CAF}, the maximal amplitude of I_{CAF} at each caffeine concentration was normalized to that of I_{CAF} evoked at 1 mM of caffeine and plotted against concentration of caffeine in Figure 2c. The concentration of caffeine for normalization was chosen to elicit a nearly half-maximal response reproducibly. I_{CAF} was detectable at 0.3 mM caffeine and increased in amplitude in a concentration-dependent manner. The quasi maximal I_{CAF} was achieved at 10 mM. When concentrations of caffeine were higher than 10 mM, current generation was less reproducible than at low concentrations even at long intervals of >10 min.

Ca^{2+}-dependence of STOCs and caffeine-responses

Since STOCs and I_{CAF} seemed to be generated by Ca^{2+}-activated K^+ channels on the basis of their pharmacological properties, we next examined the Ca^{2+}-dependence of both outward currents. Figure 3a shows the effects of a nominally Ca^{2+}-free external solution on STOCs and I_{CAF} at a V_H of -20 mV. Both outward currents were gradually suppressed in all gastric smooth muscle cells tested ($n=7$). This suggests that both STOCs and I_{CAF} need Ca^{2+} influx across the plasma membrane for their maintenance. Similarly, the normal external solution containing nicardipine, an antagonist of L-type voltage-gated Ca^{2+} channels, produced suppression of STOCs and I_{CAF} in all cells tested ($n=7$, see Figure 4a). The extent of suppression of both types of caffeine-induced effects was nearly identical in the Ca^{2+}-free and nicardipine-containing solutions although the suppression time course varies from cell to cell, presumably because of the capacity of their caffeine-sensitive Ca^{2+} stores. These results suggest that L-type voltage-gated Ca^{2+} channels participate in the generation of I_{CAF} as an important Ca^{2+} supplier.

Figures 3b and 4b show representative examples of the fura-2 microscopic measurements of $[Ca^{2+}]_i$ in responses to caffeine and the effects of the Ca^{2+}-free or nicardipine-containing external solution. Consistent with the effects on I_{CAF}, the caffeine-induced $[Ca^{2+}]_i$ elevation was suppressed in the Ca^{2+}-free or nicardipine-containing solution in all cells tested ($n=7$). Both Ca^{2+}-free and nicardipine-containing solutions produced little effect on the resting $[Ca^{2+}]_i$ in all cells tested ($n=7$).

Effects of ryanodine on STOCs and caffeine-responses

In various tissues, Ca^{2+}-activated K^+ currents evoked by caffeine are known to be triggered by Ca^{2+} release from ryanodine-sensitive Ca^{2+} stores (Golovina & Blaustein, 1997; Sugita *et al*., 1998). We next tested the effects of ryanodine on STOCs and I_{CAF}, and on the $[Ca^{2+}]_i$ elevation in response to caffeine. When ryanodine was applied at 1 μM, both outward currents were completely abolished and the effect of ryanodine was irreversible in all gastric smooth muscle cells tested ($n=6$) (data not shown). Figure 5b shows the effect of ryanodine on the caffeine-induced $[Ca^{2+}]_i$ elevation. In contrast to the transient profile of caffeine-induced $[Ca^{2+}]_i$ elevation under control conditions (Figures 3b and 4b), caffeine provoked prolonged elevations of $[Ca^{2+}]_i$ in the ryanodine-treated smooth muscle cells. The transient $[Ca^{2+}]_i$ elevation by caffeine was followed by a prolonged increase in $[Ca^{2+}]_i$ in most gastric smooth muscle cells tested ($n=5/7$ cells tested) as shown in Figure 5b. Moreover, in all ryanodine-treated smooth muscle cells ($n=7$), the caffeine-induced $[Ca^{2+}]_i$ elevation was an all-or-nothing event (data not shown) in contrast to the control responses to caffeine (see Figures 3b and 4b).

Effects of caffeine on smooth muscle contractility

Caffeine is known as a stimulant of ryanodine-sensitive Ca^{2+} release in a variety of tissues (Kimball *et al*., 1996). The effects of caffeine on muscular contraction vary, depending upon the tissue involved. In cultured murine skeletal muscle preparation (Suda & Heinemann, 1996), and guinea-pig ventricular muscle strips (Kitazawa, 1988), caffeine acted as a contractile agent, whilst, in bladder smooth muscle strips of BALB/c mice, caffeine elicited relaxation when the strips were precontracted with carbachol (CCh); although, neither contraction nor relaxation of the strips was elicited by caffeine alone (Sugita *et al*., 1998). We tested the effects of caffeine on the contractility of mouse gastric smooth muscle. Figure 6a shows a representative example of spontaneous contraction of the mouse gastric smooth muscle strips and the effects of caffeine. The spontaneous contractions occurred at a frequency of 4.90 ± 0.16 min^{-1} ($n=39$) and peak amplitude of 0.53 ± 0.02 mN ($n=39$) under control conditions. Caffeine at concentrations of >0.3 mM decreased both baseline tone and the amplitude of spontaneous contractions. When caffeine was applied at >3 mM, a single transient contraction of 0.54 ± 0.11 mN ($n=13$) was produced. The contraction was followed by a relaxation of 0.14 ± 0.05 mN ($n=10$) when measured from the baseline tone (see Figure 6a, inset).

Figure 6b shows the effects of caffeine on a smooth muscle strip precontracted with a high K^+ (50 mM) solution. The precontracted muscle strips lacked the spontaneous contraction in all strips tested ($n=8$). Caffeine elicited both contraction and relaxation in the precontracted strips in a caffeine concentration-dependent manner. The maximal relaxation was 0.41 ± 0.08 mN ($n=8$) at 3 mM caffeine, when measured from the level just before application of 3 mM caffeine. In contrast to the control conditions, caffeine elicited transient contractions even at low concentrations (0.1–0.3 mM). As shown in Figure 6b, the caffeine-induced contraction evoked at low concentrations revealed much slower kinetics than those elicited at high (>0.3 mM) concentrations of caffeine. These results suggest that the transient contraction evoked by low concentrations of caffeine in high K^+-precontracted smooth muscle strips

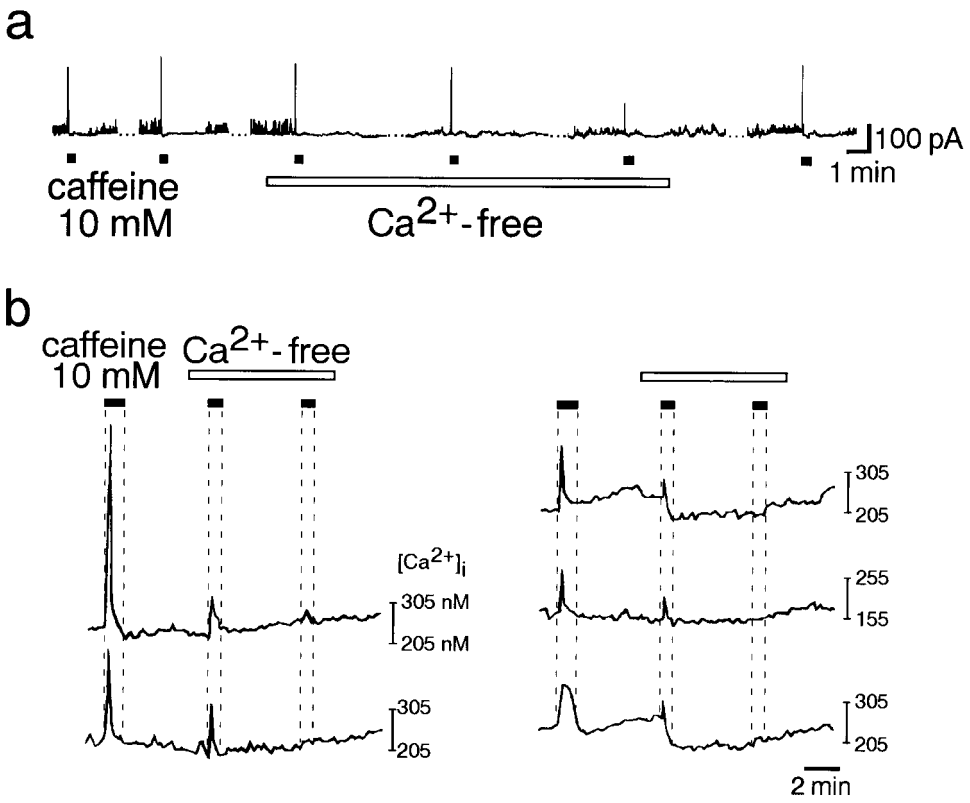

Figure 3 Effects of Ca^{2+}-free media on STOCs and caffeine-induced responses. (a) Effects of nominally Ca^{2+}-free external solutions on STOCs and I_{CAF} at a V_H of -20 mV. (b) Effects of nominally Ca^{2+}-free external solutions on fura-2 microscopic $[Ca^{2+}]_i$ signals in the response to caffeine. All traces in (b) are the records from separate gastric smooth muscle cells tested simultaneously. Horizontal closed bars indicate application of caffeine, while open bars indicate application of the Ca^{2+}-free external solution. Dotted lines indicate the zero current level.

involves different mechanisms, from that evoked by high concentrations of caffeine.

Caffeine is an inhibitor of phosphodiesterase, which gives rise to an increase in the cytoplasmic cyclic AMP level (Hall *et al*., 1990). The direct effects of cyclic AMP on the contraction may lead us to further understand the concentration-dependent effects of caffeine. We tested the effects of the membrane-permeable cyclic AMP-analogue, dibutyryl cyclic AMP (dB-cAMP), on the contractility of gastric smooth muscle strips (Figure 6c). In all smooth muscle strips tested ($n=6$), dB-cAMP mimicked the effects of low concentrations of caffeine, producing relaxation in a dB-cAMP concentration-dependent manner although dB-cAMP at >100 μM elicited a transient contraction in two out of six smooth muscle strips tested. Caffeine at >3 mM produced the transient contraction followed by the maximal relaxation in the presence of 100 μM dB-cAMP (Figure 6c). The extent of the transient contraction in the presence of 100 μM dB-cAMP was 0.28 ± 0.04 mM ($n=6$), which is significantly smaller than that of the control. This suggests that some part of the caffeine-induced transient contraction was occlusively suppressed by dB-cAMP. The maximal relaxation induced by caffeine was 0.12 ± 0.04 μN ($n=6$) after treatment with dB-cAMP when measured from the baseline tone before application of caffeine (Figure 6c). It is noteworthy that 50 mM KCl-induced contraction was significantly augmented by $162\pm33\%$ ($n=8$) after treatment with dB-cAMP when measured at the peak amplitude of contraction (see Figure 6d).

We have previously reported that bladder smooth muscle cells of BALB/c mice respond to CCh, developing a Ca^{2+}-activated K^+ current (I_{CCh}) and $[Ca^{2+}]_i$ elevation in a manner similar to those evoked by caffeine. Both I_{CCh} and CCh-induced $[Ca^{2+}]_i$ elevation of bladder smooth muscle cells were reproducible when CCh was applied repeatedly. This is completely different from the 'all-or-nothing' fashion of the CCh responses in intestinal smooth muscle cells of rat (Ohta *et al*., 1994) and guinea-pig (Iino *et al*., 1993). Figure 7a,b show representative I_{CCh} and CCh-induced $[Ca^{2+}]_i$ elevation of gastric smooth muscle cells in comparison with caffeine responses. Both I_{CCh} and CCh-induced $[Ca^{2+}]_i$ elevation were detected at CCh concentrations >1 μM. In contrast to bladder smooth muscle cells of the same BALB/c mice, those two types of single cell response to CCh occurred in an 'all-or-nothing' fashion ($n=6$ for I_{CCh} and $n=7$ for $[Ca^{2+}]_i$), similar to those of intestinal smooth muscle cells of rat (Ohta *et al*., 1994) and guinea-pig (Iino *et al*., 1993). This did not allow experiments for the concentration-response relationship for I_{CCh} and $[Ca^{2+}]_i$ elevation. In gastric smooth muscle cells in the present study, the relative I_{CCh} evoked at 10 μM CCh was 0.87 ± 0.07 ($n=6$) when normalized to that induced by 3 mM caffeine.

Figure 7c shows representative contractions of gastric smooth muscle strips to a cumulative application of CCh. In the smooth muscle strips, CCh elicited concentration-dependent contraction in all strips tested ($n=7$) with the concentration required for the half-maximal contraction

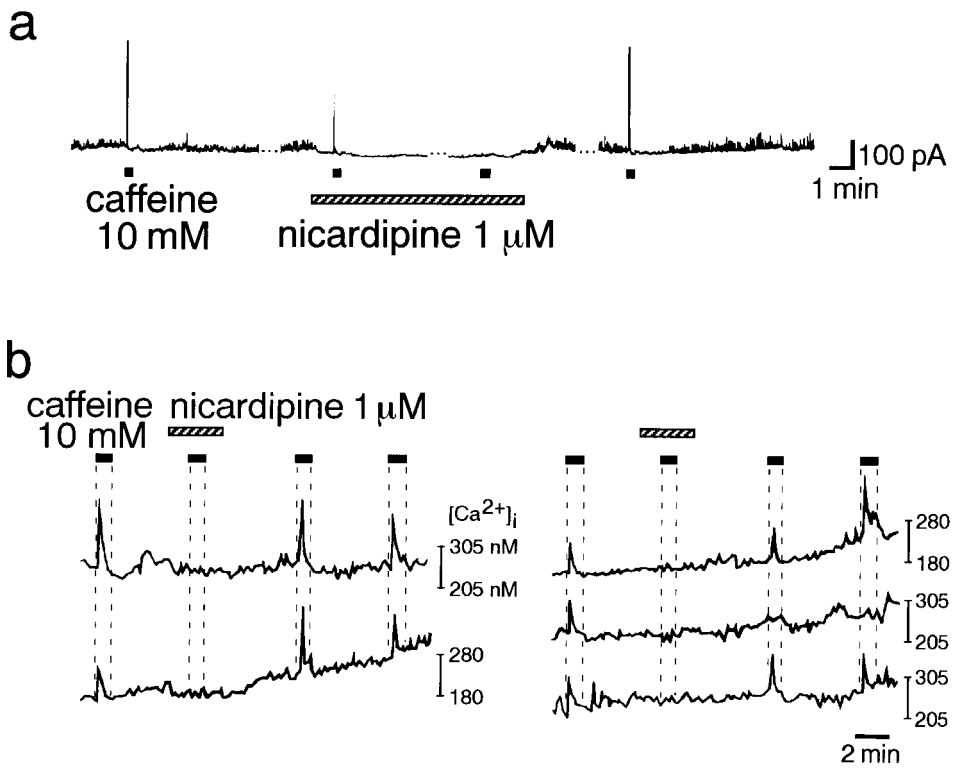

Figure 4 Effects of nicardipine-containing media on STOCs and caffeine-induced responses. (a) Effects of nicardipine-containing external solutions on STOCs and I_{CAF} at a V_H of -20 mV. (b) Effects of nicardipine-containing external solutions on fura-2 microscopic $[Ca^{2+}]_i$ signals in the response to caffeine. All traces in (b) are the records from separate gastric smooth muscle cells tested simultaneously. Horizontal closed bars indicate application of caffeine, while striped bars indicate application of nicardipine. Dotted lines indicate the zero current level.

(EC_{50}) being 0.3 μM. Caffeine produced relaxation of the CCh-contracted muscle strips in a caffeine concentration-dependent manner (not shown data). The maximal relaxation was 0.52 ± 0.07 mN ($n=7$) at caffeine concentration of 3 mM and the EC_{50} value for the caffeine relaxation was 0.55 mM when smooth muscle strips were contracted by CCh at 3 μM (data not shown).

Contraction and relaxation kinetics of high concentration caffeine-induced responses

The transient contraction as the quasi maximal response to caffeine at high concentrations (Figure 6a, inset) may reflect the functional roles and property of ryanodine-sensitive Ca^{2+} stores in muscle contraction. We next studied the contraction and relaxation kinetics of the quasi maximal response to caffeine under various conditions. Figure 8a shows representative examples of the transient contraction followed by relaxation at 3 mM caffeine under control conditions (Figure 8a, upper left panel) and TEA- (Figure 8a, upper right panel), 50 mM KCl- (Figure 8a, lower left panel) and CCh-treated conditions (Figure 8a, lower right panel). Both contraction and relaxation in the quasi maximal response to caffeine revealed single exponential kinetics under control, TEA-, IbTx- and 50 mM KCl-treated conditions. In CCh-precontracted smooth muscle strips, 3 mM caffeine produced only relaxation (see Figure 8a, lower right panel). Moreover, the caffeine-induced relaxation in the CCh-treated strips revealed double exponential kinetics. The values of time constant for the contraction (τ_C) and relaxation (τ_R) of the quasi maximal response to caffeine were presented in Figure 8b. Values of τ_C under TEA-, IbTx- and 50 mM KCl-treated conditions were not significantly different from that of the control responses. Values of τ_R under control conditions and TEA- and IbTx-treated conditions were nearly constant although the value of τ_R significantly decreased in the 50 mM KCl-precontracted strips ($P<0.05$). In CCh-precontracted smooth muscle strips, the relaxation time constant gave two values (fast and slow τ_R) in all strips tested ($n=6$). The fast τ_R was significantly smaller than that of the control ($P<0.01$) although the slow τ_R was not significantly different from that of the control.

Discussion

To determine the properties of ryanodine-sensitive Ca^{2+} storage and release in mouse gastric smooth muscle cells, we investigated membrane currents, including STOCs, I_{CAF} and I_{CCh} under voltage-clamped conditions, and $[Ca^{2+}]_i$ elevations elicited by caffeine and CCh. We also investigated the effects of caffeine and other contractile stimulants on isometric tension of the gastric smooth muscle strips. Our results indicate that ryanodine-sensitive Ca^{2+} release in the gastric smooth muscle cells of mice has great dependence upon the activity of L-type voltage-gated Ca^{2+} channels and results in activation of Ca^{2+}-activated K^+ channels. The results suggest that in regulation of the tonus of gastric smooth muscle strips, the storage of ryanodine-sensitive Ca^{2+}

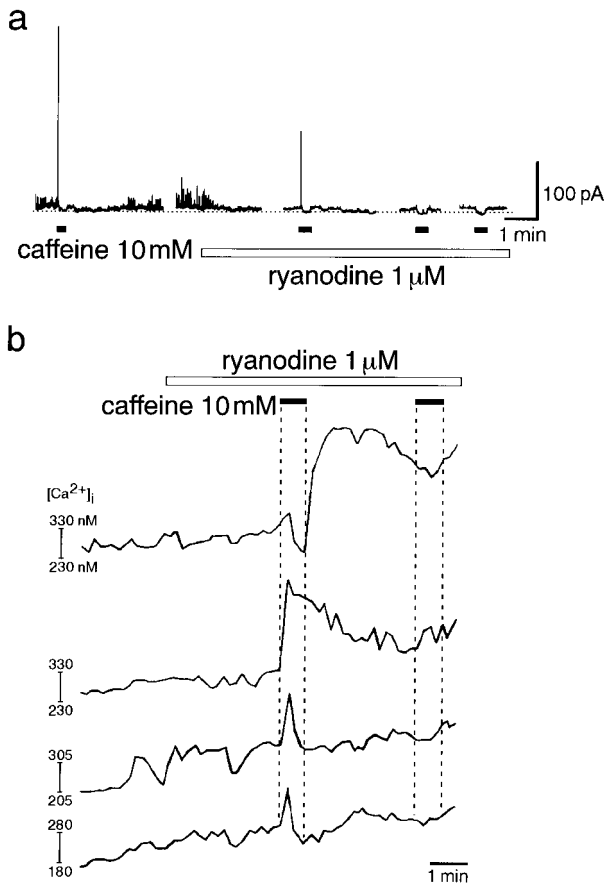

Figure 5 Effects of ryanodine on STOCs and caffeine-induced responses. (a) Effects of ryanodine on STOCs and I_{CAF} at a V_H of -20 mV. Dotted lines indicate the zero current level. (b) Effects of ryanodine on fura-2 microscopic $[Ca^{2+}]_i$ signals in the response to caffeine. All traces in (b) are the records from separate gastric smooth muscle cells tested simultaneously. Horizontal closed and open bars indicate application of caffeine and ryanodine.

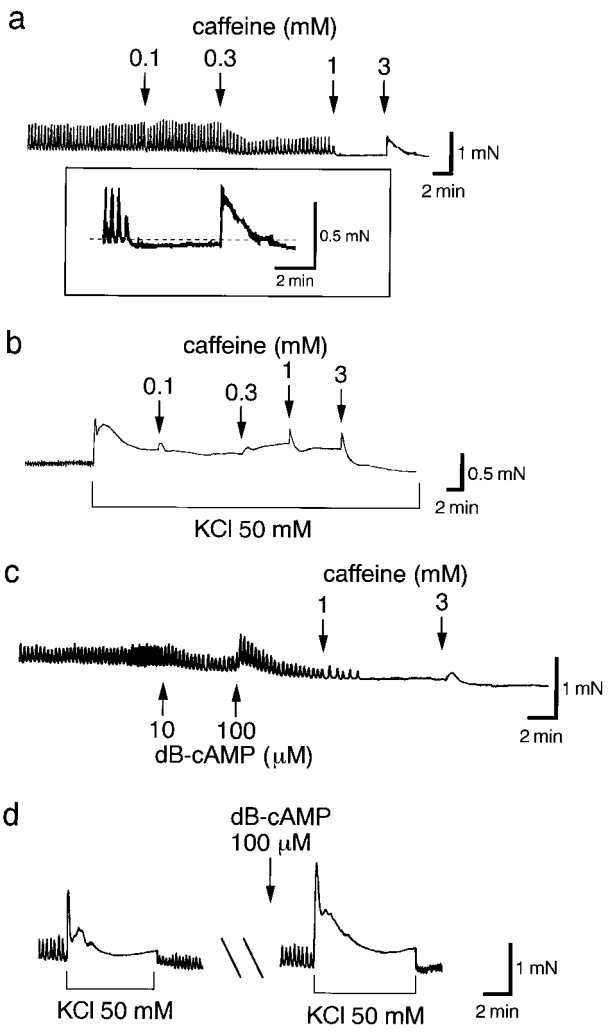

Figure 6 Responsiveness to caffeine of gastric smooth muscle strips under (a) control conditions and pretreatment conditions with a (b) 50 mM KCl-containing solution, and (c) dB-cAMP-containing solution. (d) High K^+ contraction before (left panel) and after (right panel) treatment with dB-cAMP. Inset in (a) enlarges 1 and 3 mM caffeine-induced effects. Arrows indicate cumulative application of caffeine and dB-cAMP. Fifty mM KCl was applied at indicated period.

stores interacts with other types of Ca^{2+} source for the intracellular Ca^{2+} mobilization, including $InsP_3$-sensitive Ca^{2+} release and Ca^{2+} entry through voltage-gated Ca^{2+} channels. The Ca^{2+} mobilized by ryanodine-sensitive Ca^{2+} release might participate in the modulation of function of contractile elements such as myosin light chain kinase in addition to the direct triggering of muscle contraction by the released Ca^{2+}.

The major component of STOCs and I_{CAF} seemed to be generated by Ca^{2+}-activated K^+ channels with large conductance (BK-type channels). I_{CAF} evoked at various concentrations of caffeine revealed a concentration-response relationship that was similar to those of caffeine-evoked Ca^{2+}-activated K^+ currents described in literature (Bolton & Lim, 1989; Fujii *et al*., 1997; Sugita *et al*., 1998). STOCs and I_{CAF} were greatly suppressed by TEA and IbTx but were resistant to charybdotoxin and apamin. The IbTx-sensitive but charybdotoxin-resistant nature of STOCs and I_{CAF} may characterize the BK-type channels involved in the gastric smooth muscle cells of mice. Both STOCs and I_{CAF} were highly sensitive to the concentration of extracellular Ca^{2+} ($[Ca^{2+}]_o$) and were suppressed by nicardipine, an L-type voltage-gated Ca^{2+} channel antagonist, in a manner similar to that described in the literature (Sugita *et al*., 1998). The caffeine-induced $[Ca^{2+}]_i$ elevation was also suppressed both in the Ca^{2+}-free and nicardipine-containing external solutions although the resting $[Ca^{2+}]_i$ was constant. The resting $[Ca^{2+}]_i$ might be protected against an extreme change in $[Ca^{2+}]_o$ by unknown mechanisms. For example, intracellular Ca^{2+} stores might compensate for the effect of extreme change in $[Ca^{2+}]_o$. A similar close relation between caffeine-induced Ca^{2+}-activated K^+ current and activity of L-type voltage-gated Ca^{2+} channels was also found in rat ventricular myocytes (Adachi-Akahane *et al*., 1996). Such a close relationship between cellular functions and the source of mobilized Ca^{2+} in the cytoplasm was found in skeletal muscle cells (Yamazawa *et al*., 1997). The $[Ca^{2+}]_o$-dependence and TEA- (Bolton & Lim, 1989), IbTx- (Galvez *et al*., 1990) and nicardipine-sensitivity of STOCs and I_{CAF} suggest that both currents were generated by BK-type Ca^{2+}-activated K^+ channels. Other types of Ca^{2+}-activated membrane conduc-

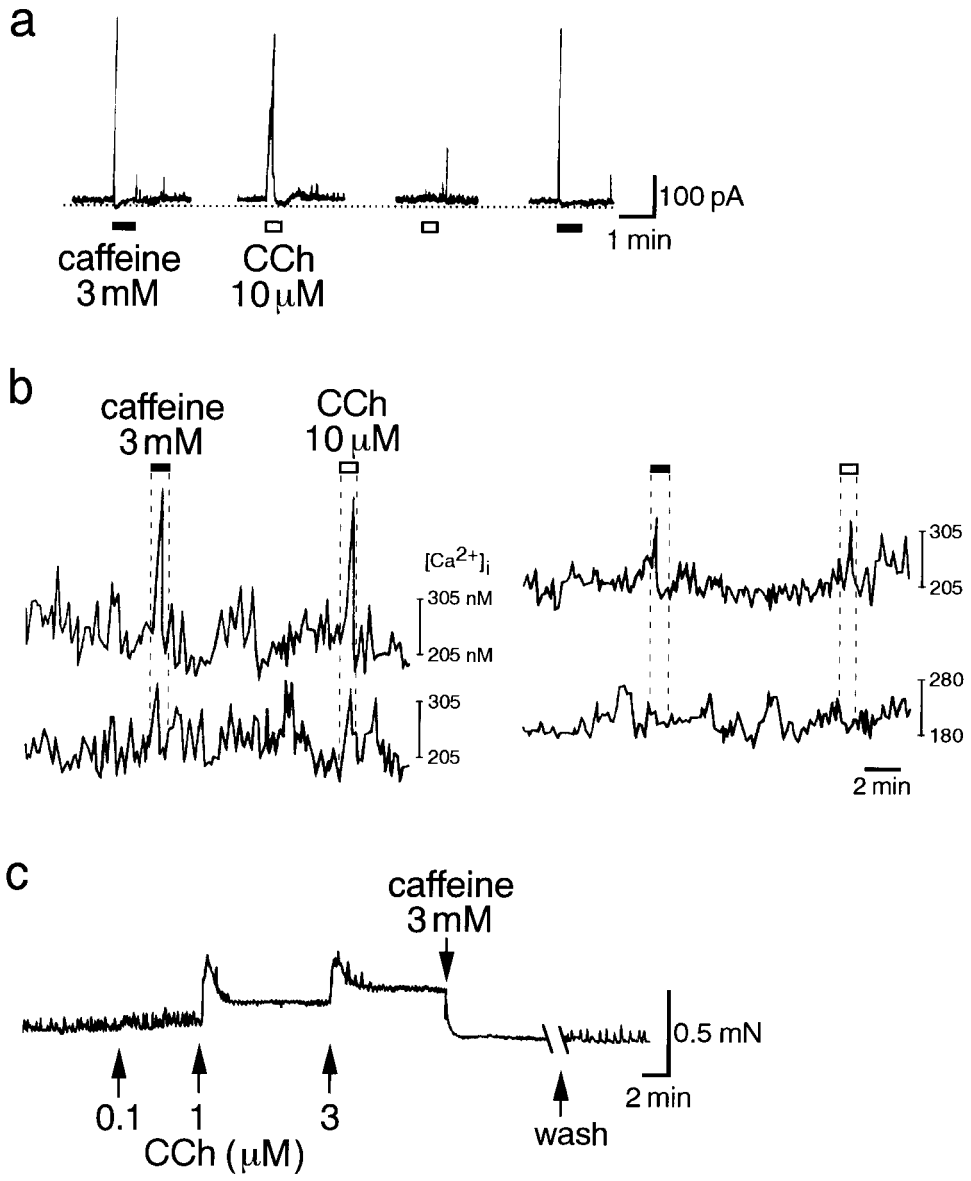

Figure 7 Difference between the effects of caffeine and CCh. (a) Caffeine- and CCh-induced outward currents at a V_H of -20 mV in gastric smooth muscle cells. (b) Caffeine- and CCh-induced $[Ca^{2+}]_i$ elevations measured with fura-2. All traces in (b) are the records from separate gastric smooth muscle cells tested simultaneously. Horizontal closed and open bars indicate application of caffeine and CCh. (c) Effects of caffeine on the contracted gastric smooth muscle with CCh. Arrows indicate cumulative applications of CCh and caffeine and removal of the drugs.

tance such as Ca^{2+}-activated Cl^- currents that were found as spontaneous transient inward currents and caffeine-induced inward currents (Hogg *et al*., 1993; Ohta *et al*., 1993) were rarely detected in the mouse gastric smooth muscle cells in our experiments (see Figure 1a).

Activation of BK channels is known to greatly depend upon the function of ryanodine-sensitive Ca^{2+} stores in rat cerebral arteries (Knot *et al*., 1998). Consistently, both I_{CAF} and caffeine-induced Ca^{2+} signals of the mouse gastric smooth muscle cells were irreversibly suppressed when the cells were incubated with ryanodine (see Figure 5). STOCs were also abolished under the same conditions. These results suggest that both Ca^{2+}-activated K^+ currents utilize the same ryanodine-sensitive Ca^{2+} release. In ryanodine-treated gastric smooth muscle cells, the caffeine-induced elevation of $[Ca^{2+}]_i$ revealed prolongation of the Ca^{2+} signal. The prolonged increase in $[Ca^{2+}]_i$ might be due to activation of store-operated Ca^{2+} entry channels (Patterson *et al*., 1999), which may be triggered by depletion of the Ca^{2+} stores (Wayman *et al*., 1998) in the presence of ryanodine and caffeine. I_{CAF} was never prolonged in the presence of ryanodine, suggesting that the prolonged components of $[Ca^{2+}]_i$ elevation did not participate in the generation of I_{CAF}.

In contrast to the caffeine responses, CCh responses of single gastric smooth muscle cells of BALB/c mice differed to those from bladder smooth muscle cells of the same animal strain. CCh responses in single gastric smooth muscle cells of mice could not be reproduced by repeated stimulation. In single smooth muscle cells from various tissues, CCh-induced responses are found to occur mainly in

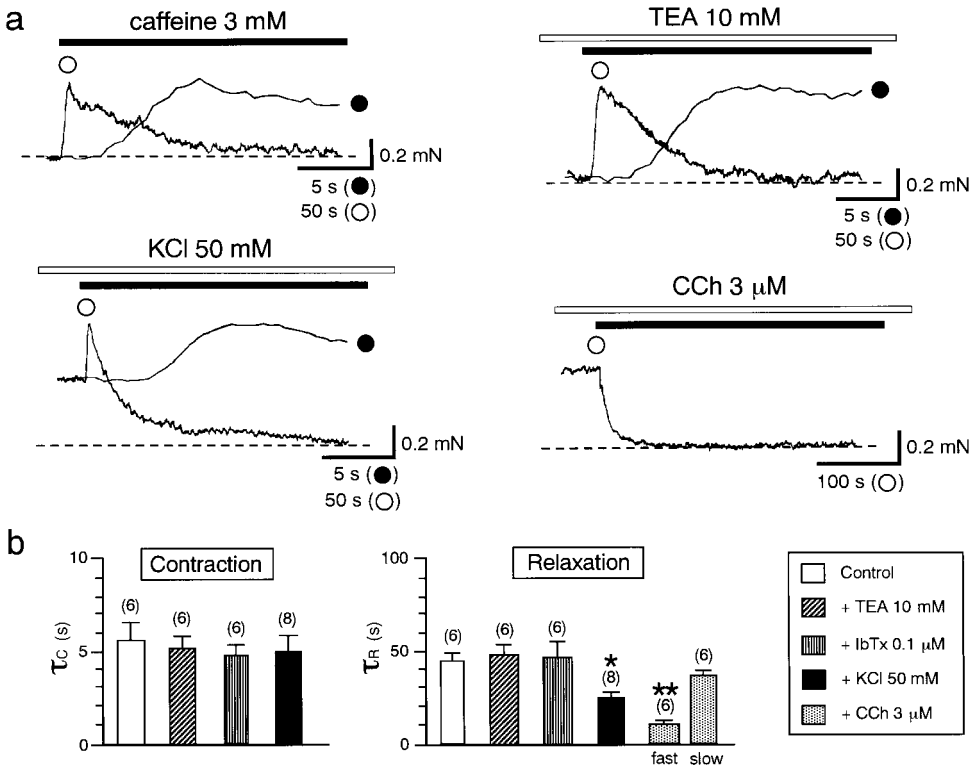

Figure 8 (a) Contraction and relaxation kinetics of high concentration (3 mM) caffeine-induced effects on the tonus under control (upper left panel), TEA (upper right panel)-, 50 mM KCl (lower left panel)- and CCh (lower right panel)-treated conditions. Horizontal closed and open bars indicate application of caffeine and other conditioning media. Dotted lines indicate the baseline. In each panel except for CCh, the same recording is presented at different time scales and both traces are superimposed. (b) τ_C and τ_R under preconditioning with TEA, IbTx, 50 mM KCl and CCh. Numerals in brackets indicate the number of strips tested. Each column represents mean ± s.e.mean. * Denotes a statistically significant decrease in the time constant (Student's unpaired *t*-test, $P<0.05$); **denotes a statistically significant decrease in the time constant (Student's unpaired *t*-test, $P<0.01$).

two distinct fashions. One is so-called 'all-or-nothing' and the other is consistently reproducible. In the 'all-or-nothing' fashion, a single smooth muscle cell responds to CCh only once, while in the reproducible fashion, the cell responds to repeated application of CCh with constant magnitude responses. In intestinal smooth muscle cells of rat (Ohta *et al*., 1994) and guinea-pig (Iino *et al*., 1993), CCh responses occurred in an 'all-or-nothing' fashion although CCh contraction of rat intestinal muscle strips reproducibly occurred in a CCh concentration-dependent manner. The concentration-dependent CCh contraction with 'all-or-nothing' fashion of single cell responsiveness was elucidated by the concentration-dependent increase in the number of responding single smooth muscle cells; the threshold for CCh varied from cell to cell (Ohta *et al*., 1994). In bladder smooth muscle cells of BALB/c mice, both I_{CCh} and the CCh-induced $[Ca^{2+}]_i$ elevation occurred in a CCh concentration-dependent manner (Sugita *et al*., 1998). Gastric smooth muscle cells of BALB/c mice in the present study revealed I_{CCh} and CCh-induced $[Ca^{2+}]_i$ elevation in an 'all-or-nothing' fashion, which is similar to that found in intestinal smooth muscle cells of rat (Ohta *et al*., 1994). Since the contraction of gastric smooth muscle strips by CCh occurred in a CCh concentration-dependent manner, the concentration-dependent CCh contraction of gastric smooth muscle strips may involve an underlying mechanism similar to that of intestinal preparations of rat (Ohta *et al*., 1994).

The caffeine action on the muscular contraction may illustrate the physiological roles of ryanodine receptors in the gastric smooth muscle cells of mice. The caffeine action is thought to involve complex mechanisms, including facilitation of Ca^{2+}-induced Ca^{2+} release, Ca^{2+}-dependent inactivation of voltage-gated Ca^{2+} channels (Nakajo *et al*., 1999), hyperpolarization caused by the generation of Ca^{2+}-activated K^+ currents (Perez *et al*., 1999), depletion of ryanodine-sensitive Ca^{2+} stores and cyclic AMP-dependent mechanisms, which may be mediated by cyclic AMP-dependent protein kinase (Stull *et al*., 1990). Those components of cellular events may separately or concomitantly result in the caffeine-induced modulation of the contractility of smooth muscle of mice, depending upon the concentrations of caffeine (see Figure 6). One important role of Ca^{2+} release from sarcoplasmic reticulum of smooth muscles is maintenance of membrane hyperpolarization mediated by Ca^{2+}-activated K^+ channels (Burdyga & Wray, 1999). However, it is unlikely that membrane hyperpolarization sustained by Ca^{2+}-activated K^+ conductance for I_{CAF} does contribute to the relaxation in response to caffeine since the caffeine relaxation was not influenced by TEA (Figure 8) at the concentrations that completely abolished I_{CAF} (Figure 2a). Another role of Ca^{2+} release in response to caffeine is triggering contraction. The transient contraction of smooth muscle strips to 3 mM caffeine (Figure 6a) may be directly triggered by the Ca^{2+} release. As shown in Figure 6c, dB-cAMP mimicked the low-

concentration of caffeine, producing relaxation of the strips. The caffeine-induced relaxation may involve a mechanism similar to that of the caffeine-induced relaxation of aortic smooth muscle strips of rat, which involved cyclic AMP-dependent pathways (Watanabe *et al*., 1992). Such a cyclic AMP-dependent mechanism is known to involve inhibitory modulation of contractile elements to activation by Ca^{2+} (Stull *et al*., 1990).

The transient contraction as the quasi maximal response to caffeine may give important information as to the role of ryanodine-sensitive Ca^{2+} stores in muscle contraction. A part of the transient contraction is thought to be due to a direct action of the Ca^{2+} released by caffeine. As shown in Figure 6a, the transient contraction was elicited at 3 mM caffeine under control conditions. While in the strips precontracted with 50 mM KCl, transient contraction was also elicited at low concentrations (0.1 – 0.3 mM) of caffeine as well as high concentrations (see Figure 6b). It is important to know if the transient contraction at low and high concentrations of caffeine has the same underlying mechanism. As shown in Figure 6d, 50 mM KCl-induced contractions were significantly augmented after treatment with dB-cAMP. This may involve cyclic AMP-dependent sensitization of the contractile elements to activation by Ca^{2+} or alternatively, potentiation of voltage-gated Ca^{2+} currents. Since the effects of dB-cAMP on muscle contractility was mainly relaxation (Figure 6c), the cyclic AMP-dependent modulation of contractile elements of gastric smooth muscle of mice is likely to result in relaxation rather than contraction, consistent with other authors (Stull *et al*., 1990; Watanabe *et al*., 1992). Thus, the augmentation of 50 mM KCl-induced contraction with dB-cAMP is more likely to be due to potentiation of voltage-gated Ca^{2+} currents (Hille, 1992). The transient contraction at low concentrations of caffeine during application of 50 mM KCl (Figure 6b) may involve the same underlying mechanism as that of the potentiated contraction with 50 mM KCl in the presence of dB-cAMP since caffeine stimulates cyclic AMP-dependent mechanism by its phosphodiesterase inhibitor action (Hall *et al*., 1990). Similarly, the transient contraction evoked by 100 μM dB-cAMP in two of six muscle strips tested might be due to potentiation of background Ca^{2+} influx through voltage-gated Ca^{2+} channels (Figure 6c). The background Ca^{2+} influx through voltage-gated Ca^{2+} channels may be consistent with the nicardipine-sensitivity of I_{CAF} and caffeine-induced $[Ca^{2+}]_i$ elevation (Figure 4). All these results suggest that the transient contraction at >3 mM caffeine involves a high magnitude of Ca^{2+} release and a potentiated Ca^{2+} influx through voltage-gated Ca^{2+} channels and that cyclic AMP-dependent mechanisms underlie the following relaxation as well as potentiating the Ca^{2+} current. Alternatively, the caffeine-induced relaxation at high concentrations of caffeine might involve depletion of stored Ca^{2+} available for contraction (Sugita *et al*., 1998).

Analysis for the contraction and relaxation kinetics for such quasi maximal responses to caffeine may give further information on the physiological roles of ryanodine receptors in the gastric smooth muscle cells of mice. Significant decrease in the relaxation time constant (τ_R) was observed in 50 mM KCl- and CCh-treated strips but not in TEA- and IbTx-treated strips (Figure 8). These results suggest that the Ca^{2+} influx through voltage-gated Ca^{2+} channels and CCh-sensitive Ca^{2+} release (Sugita *et al*., 1998) accelerate the relaxation phase in the response to high concentrations of caffeine. Contractility of tracheal smooth muscle is known to be inhibited by phosphorylation by cyclic AMP-dependent protein kinase, protein kinase C and Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), which desensitize myosin light chain kinase to activation by Ca^{2+}/calmodulin (Stull *et al*., 1990). Although it is not yet proven whether Ca^{2+} released from ryanodine-sensitive Ca^{2+} stores can activate CaMKII or Ca^{2+}/calmodulin-dependent protein phosphatase (Shaw *et al*., 1992), ryanodine is known to significantly reduce Ca^{2+}-induced contraction and phosphorylation in CCh-stimulated canine tracheal smooth muscle (Gerthoffer *et al*., 1988). Summated modulation of myosin light chain kinase might be involved in the facilitation of the caffeine-induced relaxation in the CCh-precontracted muscle strips in the present study.

In gastric smooth muscle strips precontracted with CCh, caffeine at 3 mM produced only relaxation in contrast to the caffeine response under other conditions (see Figures 7c and 8a). The lack of transient contraction by 3 mM caffeine in the CCh-treated muscle strips might be due to partial overlap between ryanodine-sensitive and $InsP_3$-sensitive Ca^{2+} stores in the cytoplasm (Golovina & Blaustein, 1997). This may support the idea that the transient contraction at >3 mM caffeine is triggered by the Ca^{2+} release from ryanodine-sensitive Ca^{2+} stores.

It is concluded that ryanodine-sensitive Ca^{2+} release in the gastric smooth muscle cells of BALB/c mice has great dependence upon the activity of L-type voltage-gated Ca^{2+} channels and results in the activation of BK-type Ca^{2+}-activated K^+ channels and that the ryanodine-sensitive Ca^{2+} storage strongly interacts with other Ca^{2+} sources, including $InsP_3$-sensitive Ca^{2+} release and Ca^{2+} entry through voltage-gated Ca^{2+} channels, in coordinating the contractility of the gastric smooth muscle of BALB/c mice. We propose that ryanodine-sensitive Ca^{2+} releasing channels, L-type Ca^{2+} channels and BK channels construct a functional unit that may modulate contractility of the gastric smooth muscle of mice in addition to the summated modulation of myosin light chain kinase. Further investigations are needed to know how the functional unit acts in abnormal mice that lack the DNA encoding a specific ryanodine receptor subtype or $InsP_3$ receptor subtype.

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