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The properties of caffeine- and carbachol-induced intracellular Ca^{2+} release in mouse bladder smooth muscle cells

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Abstract

Freshly dissociated bladder smooth muscle cells of mice developed spontaneous, caffeine- (I_{CAF}) and carbachol-induced (I_{CCh}) currents under voltage-clamped conditions. Spontaneous currents, I_{CAF} and I_{CCh} were blocked with tetraethylammonium at 3×10^{-4}–10^{-2} M but were resistant to both charybdotoxin (10^{-7}–10^{-6} M) and iberiotoxin (10^{-7}–10^{-6} M). The reversal potential for each current indicated that K^+ channels play a major role in the generation of each current. Both spontaneous currents and I_{CAF} but not I_{CCh} were abolished in nominally Ca^{2+}-free and nicardipine (10^{-6} M)-containing media. These results suggest that the activity of L-type voltage-sensitive Ca^{2+} channels is important in the generation and maintenance of spontaneous currents and I_{CAF} but not I_{CCh}. Ryanodine (10^{-6} M) prevented spontaneous currents, I_{CAF} and caffeine-induced $[Ca^{2+}]_i$ elevation but not I_{CCh} and carbachol-induced $[Ca^{2+}]_i$ elevation, suggesting that the response of bladder smooth muscle cells to carbachol may involve a Ca^{2+} store distinct from that for caffeine. Pretreatment with carbachol suppressed I_{CAF} to $22 \pm 7\%$ ($n = 7$) and the caffeine-induced $[Ca^{2+}]_i$ elevation to $25 \pm 3\%$ ($n = 6$). Similarly, caffeine suppressed I_{CCh} to $23 \pm 4\%$ ($n = 9$) and the carbachol-induced $[Ca^{2+}]_i$ elevation to $24 \pm 6\%$ ($n = 6$). © 1998 Elsevier Science B.V.

Keywords: Smooth muscle; (Mouse); Bladder; K^+ current; Ca^{2+}-activated; Patch-clamp technique; Nystatin-perforated; Fura-2 fluorometry; $[Ca^{2+}]_i$; Caffeine; Carbachol

1. Introduction

Ryanodine receptors and inositol 1,4,5-trisphosphate (IP_3) receptors are Ca^{2+}-releasing channels in the cytoplasm and play key roles in the Ca^{2+} mobilization that leads to cellular functions (Iino, 1989; Somlyo et al., 1985). The physiological and pharmacological properties of both receptors have been studied in a variety of tissues (Rousseau et al., 1986; Neering and McBurney, 1984; Berridge and Irvine, 1984). Analysis of the DNA of both receptors has revealed that at least three subtypes are involved in each receptor family and that the composition of these subtypes varies depending upon the organ involved (Takeshima, 1993; Furuichi et al., 1994). Smooth muscle tissues are relevant preparations to study the physiological significance and functional interactions of both receptors since many kinds of smooth muscle cells have both Ca^{2+}-releasing systems (Iino, 1991; Bolton and Lim, 1989).

Although it is still controversial whether both types of Ca^{2+} release are active at their specific Ca^{2+} storage sites, the resulting Ca^{2+}-dependent phenomena, such as $[Ca^{2+}]_i$ elevation, measured with Ca^{2+}-sensitive fluorescence dyes (Baro and Eisner, 1992), Ca^{2+}-activated K^+ currents (Bolton and Lim, 1989) and contractile responses (Leijten and Van Breemen, 1984) of smooth muscle preparations, seem to be distinct in their manner of occurrence and their physiological and pharmacological properties. Thus, the interaction between both receptors is an important topic for investigation.

It is also known that there is an organ- and animal species-dependent diversity in the overlap between both types of Ca^{2+}-releasing mechanism (Missiaen et al., 1992a). This overlap may due to a common Ca^{2+} storage site for both types of Ca^{2+} release. The tissue diversity

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also raises interest in extended studies of the interaction between both types of Ca^{2+} release not only under physiological conditions but also under anomalous conditions in smooth muscle.

Recent gene-targeting studies have made it possible to obtain a variety of mice that lack genes encoding specific proteins (Takeshima et al., 1994, 1996; Matsumoto et al., 1996). With this technique, both ryanodine receptor- and IP_3 receptor-deficient mice have been established (Takeshima et al., 1994, 1996; Matsumoto et al., 1996) and the features of these deficient mice have been investigated (Takeshima et al., 1994, 1996; Matsumoto et al., 1996). Several studies of both types of Ca^{2+} release in smooth muscle preparations which respond to ryanodine and IP_3 have been conducted with animals other than mice, probably because of the size of specimens. Therefore, to obtain information on the genetic background of both types of Ca^{2+} release, it is becoming important to use smooth muscle preparations from these so-called 'knock out' mice. However, little is known about the contribution of ryanodine and IP_3 receptors and their cooperation in the function of smooth muscles from standard mice. Through studies with mouse smooth muscle preparations, we found that bladder smooth muscle cells are relevant preparations to study how both types of Ca^{2+} release interact since these cells respond well to two $[Ca^{2+}]_i$-elevating stimulants, caffeine and carbachol, which respectively stimulate ryanodine-sensitive and IP_3-dependent mechanisms and generate Ca^{2+}-activated K^+ currents.

In the present study, to clarify how ryanodine-sensitive and IP_3-dependent Ca^{2+} release occurs and interacts with each other in mouse bladder smooth muscle cells, we analyzed the Ca^{2+}-activated K^+ currents and $[Ca^{2+}]_i$ elevation elicited by caffeine and carbachol and characterized the intracellular Ca^{2+} mobilization compartments involved in the responses. For measurements of the membrane current, the nystatin perforated-patch-clamp technique was used, which allows maintenance of the current responses to caffeine and carbachol.

2. Materials and methods

2.1. Cell preparations

For the experimental preparations, single smooth muscle cells from mouse bladder were freshly dissociated as described previously (Tokutomi et al., 1995) with some modifications. Briefly, BALB/c mice at day 10–15 postpartum were decapitated under ether anesthesia. The whole urinary bladder was dissected into small pieces (about 2 mmx2 mm). The fragments were incubated for 45 min in oxygenated Ca^{2+}-free external solution containing 2 mg/ml collagenase type 2 (Sigma, USA), 1 mg/ml papain (Wako, Japan), 5 mg/ml bovine serum albumin (Sigma) and 1 mg/ml trypsin inhibitor (Sigma), 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 were triturated with a pasteur pipette in the normal external solution containing 2 mM $CaCl_2$ for dissociation of single smooth muscle cells. The dissociated bladder smooth muscle cells were plated on a poly-L-lysine-coated coverslip in the center of 35-mm plastic dishes (Melidian, USA) containing 2 ml normal external solution. In 1 h, the dissociated cells became available for the patch-clamp experiments because they were strongly attached to the coverslips. The freshly dissociated smooth muscle cells were used the same day.

2.2. Recordings of membrane currents

The whole-cell recordings of membrane currents were performed with the nystatin-perforated patch-clamp technique under voltage clamp conditions through a patch-clamp amplifier (CEZ-2300, Nihon-Kohden, Japan). The data were monitored on a storage oscilloscope (5103N, Tektronix, USA) and a pen recorder (FBR-251A, Shimizu-seisakusho, Japan) and stored on a DAT recorder with custom designed modifications (DTC-59ES, Sony, Japan) for further analysis. For the nystatin-perforated patch-clamp technique, the recording patch pipette was filled with an internal solution containing nystatin at 0.1–0.3 mg/ml. This gives a pipette resistance of 2–3 MΩ in the standard external solution and the series resistance of 5–8 MΩ was compensated by $>70\%$. The advantages of the nystatin-perforated patch-clamp method have been described by several authors (Kurachi et al., 1989; Wakamori et al., 1993; Horn and Marty, 1988).

2.3. Fluorometric $[Ca^{2+}]_i$ measurements

For measurements of $[Ca^{2+}]_i$, bladder smooth muscle cells were prepared in the same way as for the electrophysiological experiments and fura-2 microscopic fluorometry was performed with an ARGUS50/CA system (Hamamatsu, Japan). Briefly, single bladder smooth muscle cells fixed on a poly-L-lysine-coated glass coverslip in the center of 35-mm plastic dishes (Melidian, USA) were incubated in the normal external solution containing 5 μM acetoxymethyl ester of fura-2 (fura-2/AM) for 20 min at 30°C in a dark room. The concentration of fura-2/AM (5 μM) was obtained from the previously described conditions (Blatter and Wier, 1992; Fujii et al., 1997). After being loaded with fura-2/AM, bladder smooth muscle cells were rinsed with normal external solution to remove the residual dye outside the cell completely and were then equilibrated for 30 min at room temperature. The fura-2-loaded bladder smooth muscle cells were illuminated by alternately exciting them at 340 and 380 nm with a xenon lamp. Fluorescent images of the bladder smooth muscle cells seen through a microscope (Nikon, DIAPHOT-TMD, Japan) were stored in an image processor (Hamamatsu,

ARGUS-50, Japan) by means of a 500-nm bandpass filter and SIT camera (Hamamatsu, C2400, Japan). The ratio of the fluorescence intensity at 340 nm excitation to that at 380 nm excitation was monitored and computer processed (Hamamatsu, U4469, Japan) to evaluate the change in $[Ca^{2+}]_i$. In both experiments, drugs were rapidly applied by use of a multibarreled pipette (Carbone and Lux, 1987) or Y-tube technique (Murase et al., 1990). All experiments were performed at 22–25°C.

2.4. Measurement of isometric tension of bladder smooth muscle

Pieces of bladder (4 mm × 4 mm) were dissected from animals aged day 10–15 postpartum and placed vertically in a 15-ml water-jacketed bath. The distal end of the piece of bladder was fixed to the bottom of the bath and the other end was fixed to a transducer for tension measurement. The tissues were bathed in the same normal external solution as used in the electrophysiological experiments. The normal external solution was gassed with 95% O_2 and 5% CO_2 and maintained at 35 ± 0.5°C. The tissues were then equilibrated for at least 30 min before the experiments. Drugs, including caffeine, carbachol and ryanodine were applied to the bath to achieve the final concentrations.

2.5. 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 internal solution contained (in mM): KCl 150 and HEPES 10. The pH of the external and internal solutions was adjusted to 7.4 with NaOH (1 M) and 7.2 with KOH (1 M), respectively. Substances used for the experiments were nystatin (Nakarai Chemical, Japan), fura-2/AM (Dojindo, Japan), caffeine (Sigma), carbachol (Sigma), nicardipine (Sigma), tetraethylammonium (Wako) and charybdotoxin (Peptide Institute, Japan), iberiotoxin (Peptide Institute, Japan), ryanodine (Wako), and thapsigargin (Wako).

The data are expressed as means ± S.E.M., and the statistical significance was estimated by Student's *t*-test. *P* values of less than 0.05 were considered to be statistically significant.

3. Results

3.1. Responsiveness of bladder smooth muscle to caffeine and carbachol

Fig. 1A shows isometric tension recorded from the same bladder smooth muscle. Open and closed bars indicate application of caffeine and carbachol, while the striped bar shows application of high K^+ (100 mM) external solution. Carbachol as well as the high K^+ solution elicited contraction in all bladder smooth muscles tested ($n = 4$). The carbachol-induced contraction occurred in a dose-dependent manner at the concentrations (10^{-6}–10^{-4} M) tested (not shown), whereas caffeine applied at $> 10^{-3}$ M did not elicit contraction in any of the smooth muscles ($n = 4$) tested (Fig. 1A, upper and middle traces). Interestingly, caffeine suppressed the carbachol-induced contraction when applied simultaneously (Fig. 1A, middle trace) although carbachol did not change the effects of caffeine (Fig. 1A, lower trace). Fig. 1B shows representative examples of caffeine (3×10^{-3} M)-induced (I_{CAF}) and carbachol (10^{-5} M)-induced currents (I_{CCh}) superimposed. Both I_{CAF} and I_{CCh} were recorded under voltage-clamped conditions at 0 mV with the nystatin-perforated patch-clamp technique. The times for the half-maximal activation ($T_{A1/2}$) and decay ($T_{D1/2}$) of I_{CAF} were 0.70 ± 0.11 and 1.03 ± 0.11 s (mean ± S.E.M., $n = 18$), respectively, when currents with a similar size were normalized, whereas the values of $T_{A1/2}$ and $T_{D1/2}$ for I_{CCh} were 0.22 ± 0.02 and 0.39 ± 0.07 s (mean ± S.E.M., $n = 8$), respectively. I_{CCh} had faster activation and decay kinetics than I_{CAF} in all bladder smooth muscle cells tested although the order of the magnitude of both currents varied from one cell to another.

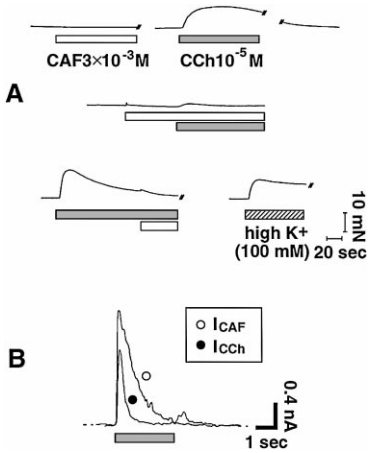

Fig. 1. Responsiveness of mouse bladder smooth muscles to caffeine and carbachol. (A) A representative example of the isometric tension of bladder smooth muscles in response to caffeine (CAF, open bars), carbachol (CCh, shaded bars) and high K^+-depolarization (a striped bar). (B) Caffeine- (I_{CAF}, open circle) and carbachol-induced outward currents (I_{CCh}, closed circle) in a freshly dissociated bladder smooth muscle cell under voltage-clamped conditions with the nystatin-perforated patch-clamp technique. The cell was voltage-clamped at 0 mV.

3.2. Spontaneous and caffeine- and carbachol-induced outward currents of bladder smooth muscle cells

The resting membrane voltage of bladder smooth muscle cells was −50.1 ± 2.8 mV (mean ± S.E.M., $n = 5$) under current–clamp conditions and the resting $[Ca^{2+}]_i$,

obtained from the fluorescence ratio ($F_{340/380}$), was 1.00 $\pm$ 0.03 (mean $\pm$ S.E.M., $n = 50$) when measured with fura-2 fluorometry.

Under voltage–clamp conditions at > -50 mV with the nystatin perforated-patch-clamp technique, most bladder smooth muscle cells ($n = 98/102$) developed spontaneous outward currents (Figs. 2–6). The rate of occurrence of spontaneous outward currents was 18.0 ± 2.2 min^{-1} (mean $\pm$ S.E.M., $n = 5$) at -20 mV and increased at higher V_H (see Fig. 2A, left). Moreover, spontaneous outward currents were inactive at V_H values of < -60 mV. The zero current levels for spontaneous outward currents, I_{CAF} and I_{CCh} are indicated by dotted lines and were obtained by application of 10^{-2} M tetraethylammonium. When the rate of occurrence of spontaneous outward currents was comparatively low, the baseline current was easily determined without application of tetraethylammonium and was not affected with tetraethylammonium. The three types of outward currents, including the spontaneous outward currents, I_{CAF} and I_{CCh}, were used as indicators of cytoplasmic Ca^{2+} mobilization. Both I_{CAF} ($n = 81/89$) and I_{CCh} ($n = 62/76$) were observed in most bladder smooth muscle cells tested. I_{CAF} was monophasic, while I_{CCh} was a multiphasic oscillatory current (see Fig. 2B, upper traces) in nearly half of the carbachol-sensitive bladder smooth muscle cells ($n = 37/62$). The rest of the carbachol-sensitive bladder smooth muscle cells had a monophasic I_{CCh}. I_{CCh} was elicited at 10^{-6}–10^{-4} M of carbachol. The rate of oscillation of I_{CCh} at 10^{-5} M in the oscillatory-type bladder smooth muscle cells was 24.6 ± 3.0 min^{-1} (mean $\pm$ S.E.M., $n = 10$) and was nearly constant for the concentrations of carbachol (10^{-6}–10^{-4} M) tested. Fig. 2 shows representative examples of spontaneous outward currents (Fig. 2A, left traces), I_{CAF} and I_{CCh} (Fig. 2B, upper traces) and their current–voltage (I–V) relation curves (Fig. 2A, right panel and B, lower panel). The amplitude of spontaneous outward currents was measured by averaging the current recordings sampled for 1 min at each V_H. The maximal amplitude for

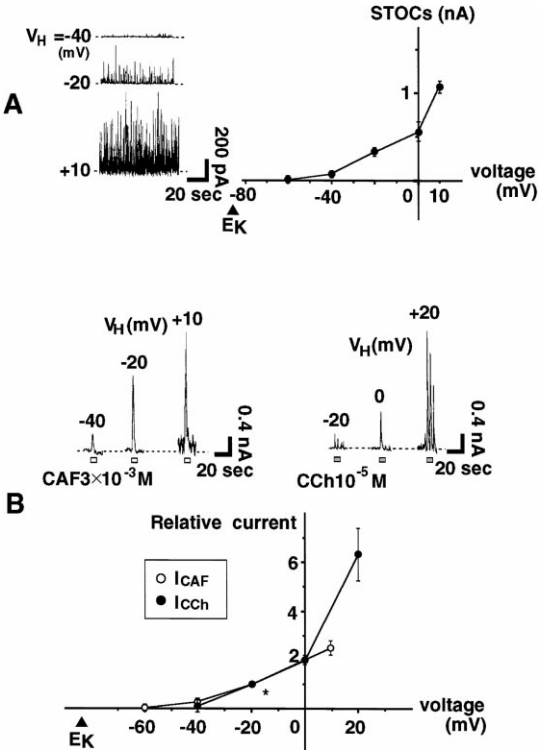

Fig. 2. Current–voltage (I–V) relationships for spontaneous outward currents, I_{CAF} and I_{CCh}. (A) A representative example of spontaneous outward currents (left) and the I–V relation curve for spontaneous outward currents (STOCs) (right). Symbols in the I–V curve indicate mean amplitude and S.E.M. of spontaneous outward currents from bladder smooth muscle cells ($n = 5$). (B) Representative examples of I_{CAF} (upper, left)and I_{CCh} (upper, right) and their I–V relation curves (lower panel). Open and shaded bars indicate application of caffeine (CAF) and carbachol (CCh), respectively. Symbols in the I–V curves indicate mean amplitude and S.E.M. of I_{CAF} (open circle) and I_{CCh} (closed circle) from twelve bladder smooth muscle cells for I_{CAF} and ten cells for I_{CCh}. The amplitude of each current was normalized to the control of each current at -20 mV (asterisk). Dotted lines in (A) and (B) indicate the zero current level.

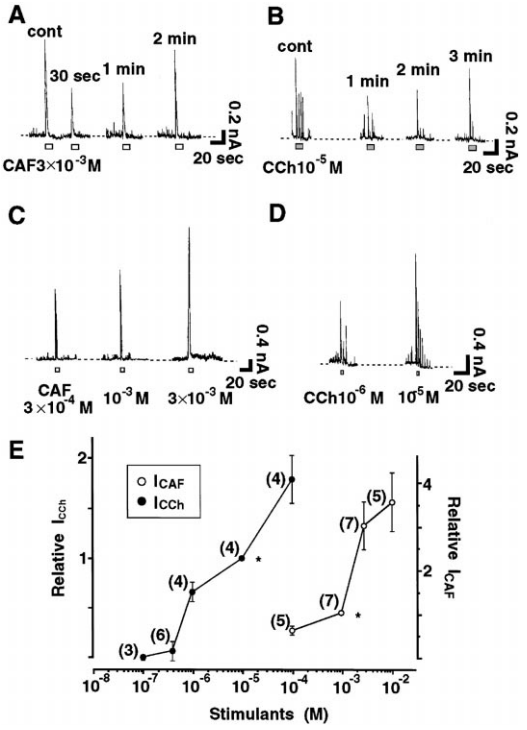

Fig. 3. Recovery and concentration–response relationships of I_{CAF} and I_{CCh}. Recovery of I_{CAF} (A) and I_{CCh} (B), tested at indicated intervals at a V_H of 0 mV. Open and shaded bars indicate application of caffeine (CAF) and carbachol (CCh), respectively. For testing the dose–response relationships, I_{CAF} (C) and I_{CCh} (D) were elicited at various concentrations of caffeine (CAF) and carbachol (CCh). (E) Concentration–response curves for I_{CAF} and I_{CCh}. I_{CAF} (open circle) was normalized to the amplitude of I_{CAF} at 10^{-3} M, while I_{CCh} (closed circle) was normalized to the amplitude of I_{CCh} at 10^{-5} M, as indicated with asterisks. Symbols in the curves indicate mean values and S.E.M. Numerals in parentheses indicate number of cells tested. Dotted lines in each panel of the current recordings indicate the zero current level.

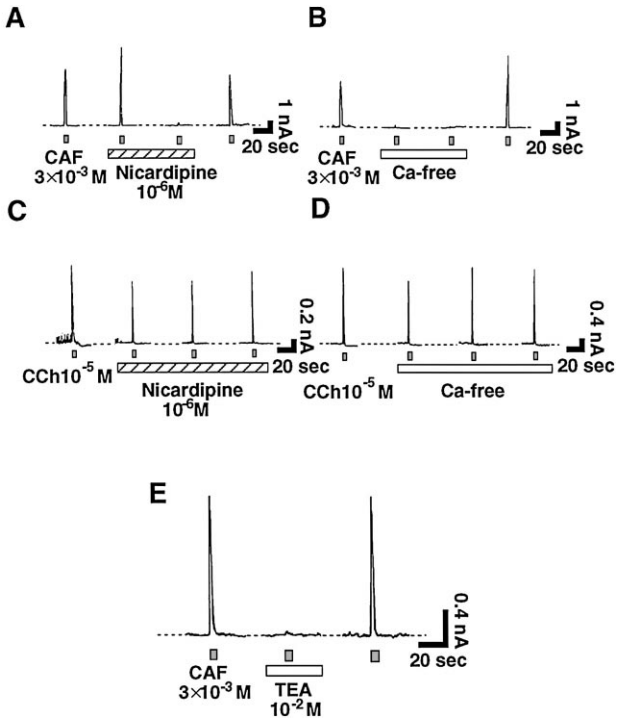

Fig. 4. Ca^{2+}-dependence and pharmacological properties of I_{CAF} and I_{CCh}. (A) and (B) Effects of nicardipine (striped bar) and nominally Ca^{2+}-free external solution (open bar) on I_{CAF} at a V_H of 0 mV. Shaded bars indicate application of caffeine (CAF). (C) and (D) Effects of nicardipine (striped bar) and nominally Ca^{2+}-free external solution (open bar) on I_{CCh} at 0 mV. Shaded bars indicate application of carbachol (CCh). (E) Effects of tetraethylammonium (TEA) on I_{CAF} at -20 mV. Shaded and open bars indicate application of caffeine and tetraethylammonium. Dotted lines in each panel of the current recordings indicate the zero current level.

I_{CAF} and I_{CCh} was measured at each V_H and was normalized to the value at -20 mV. The I–V curve for spontaneous outward currents was obtained from five bladder smooth muscle cells, while those for I_{CAF} and I_{CCh} were from 12 and 10 bladder smooth muscle cells, respectively. I–V curves for the three types of outward currents revealed outward rectification, similar to that described for Ca^{2+}-activated K^+ currents of rabbit intestinal smooth muscle cells (Benham and Bolton, 1986) and bovine ciliary muscle cells (Fujii et al., 1997). As shown in Fig. 2A, extrapolation of the I–V curve for spontaneous outward currents revealed a reversal potential (V_R) of nearly -80 mV. That is close to the equilibrium potential for K^+ (E_K), which was calculated as -85 mV by using the Nernst equation with $[K^+]_o$ and $[K^+]_i$ being 5 and 150 mM. Similarly, the I–V curves for both I_{CAF} and I_{CCh} (Fig. 2B) indicated that the major component of the membrane conductance underlying I_{CAF} and I_{CCh} is a K^+ conductance.

3.3. Recovery and dose-dependence of I_{CAF} and I_{CCh}

The time for recovery of I_{CAF} and I_{CCh} was compared by changing the application intervals of each stimulant. I_{CAF} was evoked at 3×10^{-3} M of caffeine, while I_{CCh} was evoked at 10^{-5} M of carbachol since these submaximal concentrations allowed reproducible responses to both stimulants applied for 10 s. When caffeine was applied subsequently after 30 s, the second I_{CAF} was decreased in amplitude by nearly 50% of the control I_{CAF} (see Fig. 3A). When the interval was increased, I_{CAF} gradually increased in amplitude, reaching the control level (see Fig. 3A). Fig. 3B shows the recovery time-course of I_{CCh}, which was examined with the same experimental protocol as that for I_{CAF}. I_{CCh} had a similar procedure of recovery to that of I_{CAF}. I_{CCh} took much longer to recover than I_{CAF}. On average, maximal recovery was achieved at 3 min for I_{CAF} ($n=7$) and at 5 min for I_{CCh} ($n=9$). Both I_{CAF} and I_{CCh} showed no desensitization when elicited repeatedly at intervals long enough to allow recovery of both currents.

Fig. 3C and D show I_{CAF} and I_{CCh} at various concentrations of caffeine and carbachol. I_{CAF} was tested at a V_H of -20 mV and I_{CCh} at 0 mV. Each V_H allowed reproducible I_{CAF} or I_{CCh} when elicited repeatedly. Each stimulant was applied for long (3–7 s) enough to observe activation and decay of the current. To obtain the concentration–response relation for the caffeine-induced current, the maximal amplitude of I_{CAF} at each caffeine concentration was normalized to that of I_{CAF} at 10^{-3} M and plotted against concentrations of caffeine in Fig. 3E (open circle), and that of I_{CCh} was normalized to the maximal current amplitude at 10^{-5} M carbachol. The concentration of each stimulant for normalization was chosen to elicit a nearly half-maximal, reproducible response. I_{CAF} was elicited at $>10^{-4}$ M and increased in amplitude in a dose-dependent manner. The quasi-maximal I_{CAF} was achieved at $>10^{-2}$ M. I_{CCh} was detectable at 3×10^{-7} M of carbachol and increased in amplitude in a dose-dependent manner. The quasi-maximal I_{CCh} was achieved at 10^{-4} M. At high concentrations ($>10^{-2}$ M for I_{CAF} and $>10^{-4}$ M for I_{CCh}), current generation was less reproducible than at low concentrations even at long intervals of >5 min.

3.4. Pharmacological properties and Ca^{2+}-dependence of I_{CAF} and I_{CCh}

As the distinct recovery time (Fig. 3A and B) and kinetics (Fig. 1B) of I_{CAF} and I_{CCh} implied distinct intracellular events for the generation of the two currents, we examined their Ca^{2+}-dependence. Fig. 4B and D show the effects of a nominally Ca^{2+}-free external solution on I_{CAF} and I_{CCh}. When elicited in the Ca^{2+}-free solution, I_{CAF} was completely abolished in all bladder smooth muscle cells tested (Fig. 4B, $n=7$), whereas I_{CCh} was nearly resistant under the same Ca^{2+}-free conditions in all bladder smooth muscle cells tested (Fig. 4D, $n=5$). This suggests that I_{CAF} but not I_{CCh} needs the Ca^{2+}-influx from the extracellular solution. Similarly, in the normal external solution containing nicardipine at 10^{-6} M, I_{CAF} was

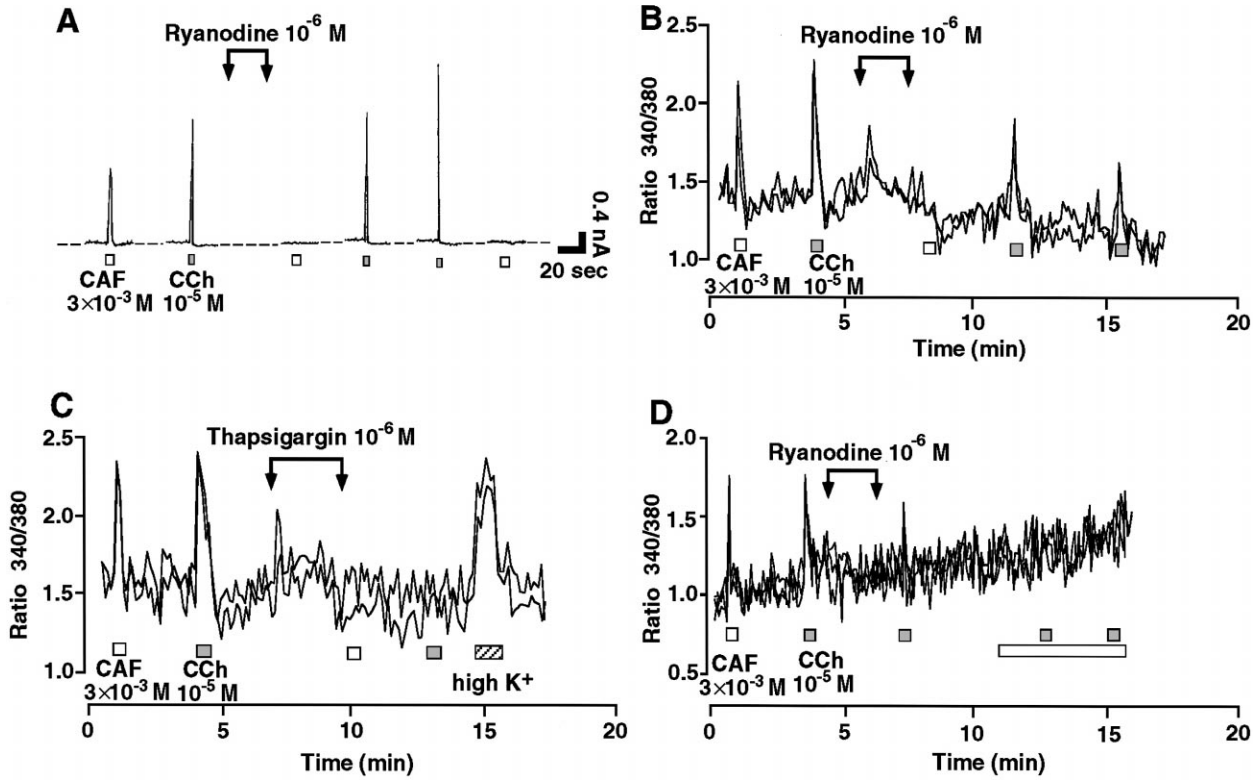

Fig. 5. Properties of caffeine- and carbachol-induced increase in $[Ca^{2+}]_i$. (A) Effects of ryanodine (arrows) on I_{CAF} and I_{CCh} at a V_H of 0 mV. Open and shaded bars indicate application of caffeine (CAF) and carbachol (CCh), respectively. Dotted lines indicate the zero current level. (B) and (D) Effects of ryanodine (arrows) on the caffeine- and carbachol-induced increase in $[Ca^{2+}]_i$. Open and shaded bars indicate application of caffeine and carbachol. Note that simultaneously applied caffeine suppressed the carbachol response without eliciting a response itself after treatment with ryanodine. (C) Effects of thapsigargin (arrows) on the caffeine- and carbachol-induced increase in $[Ca^{2+}]_i$. Open, shaded and striped bars indicate application of caffeine, carbachol and the high K^+ (100 mM) external solution.

nearly abolished (Fig. 4A, $n = 7$), but I_{CCh} was not (Fig. 4C, $n = 5$), suggesting that generation of I_{CAF} but not I_{CCh} is highly dependent upon the activity of L-type voltage-sensitive Ca^{2+} channels.

In vascular (Ganitkevich and Isenberg, 1990) and intestinal smooth muscle cells (Bolton and Lim, 1989), both spontaneous outward currents and I_{CAF} have been reported to be tetraethylammonium-sensitive Ca^{2+}-activated K^+ currents. We studied the effects of blockers of Ca^{2+}-activated K^+ channels. Fig. 4E shows a representative example of the effect of tetraethylammonium on I_{CAF}. I_{CAF} was completely blocked with tetraethylammonium at 10^{-2} M in all bladder smooth muscle cells tested ($n = 3$). Both spontaneous outward currents ($n = 3$) and I_{CCh} ($n = 3$) were similarly blocked with tetraethylammonium at the same concentration (data not shown). The tetraethylammonium block of the currents was observed at 3×10^{-4} M tetraethylammonium for both I_{CAF} and I_{CCh} and the extent of block increased in a tetraethylammonium concentration-dependent manner (data not shown). The tetraethylammonium concentration-dependence for the block of both currents was nearly identical when tested against I_{CAF} induced by 3×10^{-3} M caffeine and against I_{CCh} induced by 10^{-5} M carbachol. Neither I_{CAF}, I_{CCh} nor spontaneous outward currents were blocked by charybdotoxin or iberiotoxin at 10^{-7} to 10^{-6} M in all bladder smooth muscle cells tested (data not shown, $n = 3$ for each toxin). These results suggest that tetraethylammonium-sensitive but charybdotoxin- and iberiotoxin-resistant Ca^{2+}-activated K^+ channels are involved in each current.

3.5. Properties of Ca^{2+} stores for I_{CAF} and I_{CCh}

It is known that ryanodine leaves the ryanodine receptor open and that application of ryanodine with caffeine results in depletion of the ryanodine-sensitive Ca^{2+} stores (Iino et al., 1988). Fig. 5A, B and D show the effects of ryanodine (10^{-6} M) on I_{CAF} and I_{CCh} and on the $[Ca^{2+}]_i$-increase in response to caffeine and carbachol applied at intervals of > 5 min. I_{CAF} was abolished in the presence of ryanodine (Fig. 5A) in all bladder smooth muscle cells tested ($n = 7$), while I_{CCh} was well sustained in all bladder smooth muscle cells tested ($n = 5$) under the ryanodine-treated conditions in which I_{CAF} was completely abolished. Fig. 5B shows the caffeine- and carbachol-induced $[Ca^{2+}]_i$ elevation measured by fura-2 fluorometry. The caffeine-induced $[Ca^{2+}]_i$ elevations were abolished by treatment with ryanodine, while carbachol-induced $[Ca^{2+}]_i$

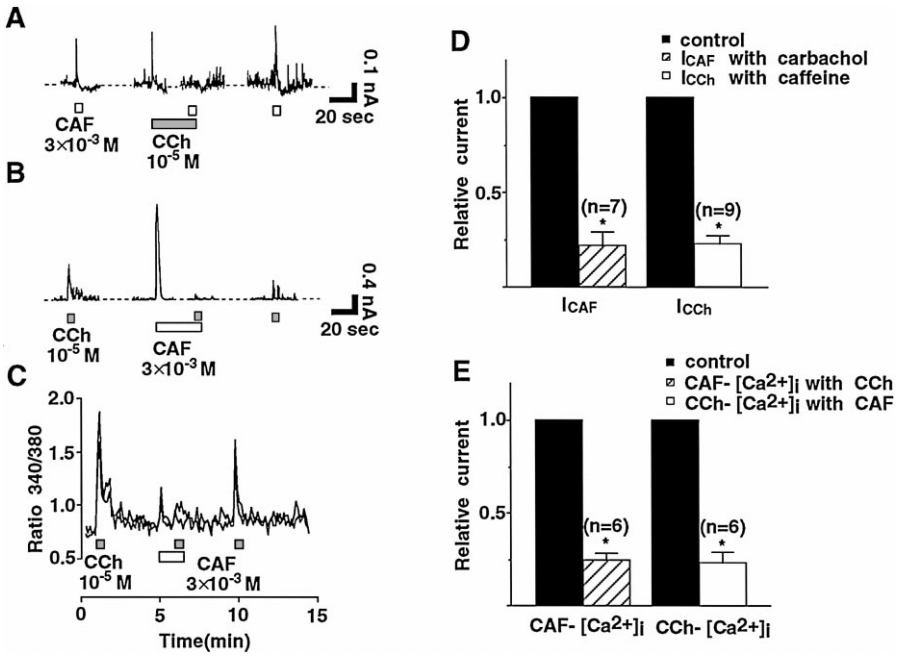

Fig. 6. Interaction between caffeine- and carbachol-induced responses. (A) Effects of simultaneous application of carbachol on I_{CAF} at a V_H of 0 mV. Open and shaded bars indicate application of caffeine (CAF) and carbachol (CCh). Dotted lines indicate the zero current level. (B) Effects of simultaneous application of caffeine on I_{CCh} at 0 mV. (C) Effects of simultaneous application of caffeine on the carbachol-induced increase in $[Ca^{2+}]_i$. (D) Interaction between I_{CAF} and I_{CCh}. Ordinate: relative amplitude of I_{CAF} and I_{CCh} normalized to that of each control (closed column). Striped and open columns indicate I_{CAF} in the presence of carbachol (I_{CAF} with carbachol) and I_{CCh} in the presence of caffeine (I_{CCh} with caffeine), respectively. Each column represents the mean ± S.E.M. (vertical line) from 7–9 experiments. (E) Interaction between caffeine- and carbachol-induced increase in $[Ca^{2+}]_i$. Ordinate: relative values of ratiometric $[Ca^{2+}]_i$ normalized to that of the control (closed column) of each stimulant response. Striped and open columns indicate caffeine-induced $[Ca^{2+}]_i$ elevation in the presence of carbachol (CAF-$[Ca^{2+}]_i$ with CCh) and carbachol-induced $[Ca^{2+}]_i$ elevation in the presence of caffeine (CCh-$[Ca^{2+}]_i$ with CAF), respectively. Each column represents the mean ± S.E.M. (vertical line) from six experiments. * Statistical significance < 0.05 in (D) and (E).

elevations were comparatively resistant to ryanodine in all bladder smooth muscle cells tested ($n = 5$). It is of interest that caffeine suppressed the carbachol-induced elevations of $[Ca^{2+}]_i$ even after the caffeine-induced response was completely abolished with ryanodine (Fig. 5D).

Fig. 5C shows the effect of thapsigargin, an inhibitor of the ATP-dependent Ca^{2+} pump. Superfusion of bladder smooth muscle cells with thapsigargin gradually abolished both I_{CAF} and I_{CCh} (data not shown). The $[Ca^{2+}]_i$ elevations evoked with caffeine or carbachol were also abolished by thapsigargin although the $[Ca^{2+}]_i$ elevation evoked by depolarization with the high K^+ (100 mM) external solution was not affected (Fig. 5C).

3.6. Interaction between responses to caffeine and carbachol

The distinct dependence upon extracellular Ca^{2+} and the ryanodine-sensitivity of I_{CAF} and I_{CCh} and the $[Ca^{2+}]_i$ signals induced by both stimulants suggested that caffeine and carbachol may act on distinct stores of intracellular Ca^{2+}. There is therefore a question whether the Ca^{2+} release in response to caffeine or carbachol depends upon a common Ca^{2+} store. To answer this question, the interaction between I_{CAF} and I_{CCh} and that between $[Ca^{2+}]_i$ elevations evoked by both stimulants were examined. Fig. 6A shows the effect of carbachol on I_{CAF}. When caffeine was applied at 5-min intervals, pretreatment with carbachol suppressed I_{CAF} though the application interval was long enough for the recovery of I_{CAF}, as shown in Fig. 3A. Pretreatment with caffeine also suppressed I_{CCh} elicited at 5-min intervals, as shown in Fig. 6B. Fig. 6D summarizes the results of the interaction between I_{CAF} and I_{CCh}. The amplitudes of I_{CAF} and I_{CCh} were normalized to those of the control I_{CAF} and I_{CCh}. After pretreatment with carbachol, I_{CAF} was suppressed to 22 ± 7% (mean ± S.E.M., $n = 7$, striped column on left in Fig. 6D), while I_{CCh} was suppressed to 23 ± 4% (mean ± S.E.M., $n = 9$, open column on right in Fig. 6D) after pretreatment with caffeine. Consistently, pretreatment with carbachol significantly suppressed the $[Ca^{2+}]_i$ elevations in the response to caffeine and pretreatment with caffeine suppressed the carbachol-induced $[Ca^{2+}]_i$ elevations (Fig. 6C and 6E). As summarized in Fig. 6E, caffeine- and carbachol-induced $[Ca^{2+}]_i$ elevations were suppressed to 25 ± 3% (mean ± S.E.M., $n = 6$) and 24 ± 6% (mean ± S.E.M., $n = 6$), respectively, by pretreatment with the accompanying stimulant when data were normalized to the respective control $[Ca^{2+}]_i$ elevations.

4. Discussion

To determine the standard properties of intracellular Ca^{2+} release in mouse bladder smooth muscle cells, membrane currents, including spontaneous, caffeine-induced (I_{CAF}) and carbachol-induced (I_{CCh}) outward currents under voltage-clamped conditions, and $[Ca^{2+}]_i$ elevations elicited by caffeine and carbachol were investigated. The major component of spontaneous outward currents, I_{CAF} and I_{CCh} was K^+ conductance since the reversal potential (V_R) for each current was close to the equilibrium potential for K^+ (E_K) (Fig. 2A and B). Spontaneous outward currents, I_{CAF} and I_{CCh} were suppressed by tetraethylammonium to a great extent but were resistant to both charybdotoxin and iberiotoxin, suggesting that each current is mediated by tetraethylammonium-sensitive K^+ channels. Both spontaneous outward currents and I_{CAF} were highly sensitive to the concentration of extracellular Ca^{2+} ($[Ca^{2+}]_o$) and were greatly suppressed by nicardipine, an L-type Ca^{2+} channel antagonist (see Fig. 4A–C). The $[Ca^{2+}]_o$ dependence and tetraethylammonium and nicardipine sensitivity of spontaneous outward currents and I_{CAF} suggest that both currents were generated by Ca^{2+}-activated K^+ channels with large conductance (BK-type channels), as described in the literature (Benham and Bolton, 1986; Inoue et al., 1985). The similar $[Ca^{2+}]_i$ elevations in response to caffeine and carbachol and the identical tetraethylammonium sensitivity of both I_{CAF} and I_{CCh} suggest that I_{CCh} involves Ca^{2+}-activated K^+ channels similar to those of I_{CAF}. The charybdotoxin- and iberiotoxin-resistance of each Ca^{2+}-activated K^+ current in the present study may imply that Ca^{2+}-activated K^+ channels in mouse bladder smooth muscle cells belong to subtypes distinct from those found in bovine aortic smooth muscle cells, which are known to be highly sensitive to iberiotoxin (Giangiacomo et al., 1992). In intestinal smooth muscle cells, a muscarinic receptor-operated nonspecific cationic current (Inoue and Isenberg, 1990) has been found. A Ca^{2+}-dependent Cl^- current in vascular smooth muscle cells of rabbit is known to be mediated by muscarinic receptors (Amédée et al., 1990). However, the $I–V$ relation curve for I_{CCh} and resistance of I_{CCh} to the Ca^{2+}-free condition suggested that the contribution of such conductances to the outward I_{CCh} in this study is very small. The concentration–response curve for I_{CAF} (Fig. 3E) revealed a dose–response relationship as described in the literature (Bolton and Lim, 1989; Fujii et al., 1997). However, single mouse bladder smooth muscle cells in this study responded to carbachol in a dose-dependent manner but not in the all-or-nothing fashion that has been found in intestinal smooth muscle cells of rat (Ohta et al., 1994) and guinea pig (Iino et al., 1993). The dose-dependent contraction of rat intestinal smooth muscles elicited by carbachol was evidenced by the dose-dependent increase in the number of responding single smooth muscle cells; the threshold for carbachol varied from cell to cell (Ohta et al., 1994). The dose-dependent responsiveness of single bladder smooth muscle cells to carbachol in the present study may characterize the IP_3-dependent Ca^{2+} mobilization in this cell type. It remains unknown whether mouse bladder smooth muscle cells have unitary events of a similar all-or-nothing type (Iino et al., 1993; Ohta et al., 1994) at a cytoplasmic level. Further studies are required to answer this question. I_{CCh} had slower recovery kinetics than I_{CAF} (Fig. 3A and B). Moreover, I_{CAF} had much slower activation and decay kinetics than I_{CCh} (Fig. 1B). These results raise the idea that I_{CCh} depends upon a source of $[Ca^{2+}]_i$ release distinct from that of I_{CAF}.

The effects of the nominally Ca^{2+}-free external solution or an L-type Ca^{2+} channel blocker, nicardipine, on both I_{CAF} and I_{CCh} are also good ways to characterize the source of Ca^{2+} release involved in the responses to caffeine and carbachol. As shown in Fig. 4A and B, I_{CAF} was abolished in the Ca^{2+}-free external solution and in the normal external solution containing nicardipine, but I_{CCh} was resistant to both conditions. These results suggest that the caffeine-induced increase in $[Ca^{2+}]_i$ depends more on Ca^{2+} influxes through L-type voltage-sensitive Ca^{2+} channels than the carbachol-induced increase in $[Ca^{2+}]_i$ does. There might be a difference in the localization of stimulated Ca^{2+} storage sites between caffeine- and carbachol-induced responses (Somlyo and Somlyo, 1994; Yamazawa et al., 1992). One can argue that nonspecific cationic conductance mediated by muscarinic receptor activation (Inoue and Isenberg, 1990) may compensate for the interrupted Ca^{2+} influx through voltage-sensitive Ca^{2+} channels in the response to carbachol under conditions where voltage-sensitive Ca^{2+} channels were blocked with nicardipine. However, this was not the case in this study since the nominally Ca^{2+}-free external solution did not prevent I_{CCh}. Moreover no trace of such a muscarinic inward current was observed under the experimental conditions used.

There were a great difference between the effect of ryanodine on I_{CAF} and that on I_{CCh} and on the $[Ca^{2+}]_i$ elevation evoked with caffeine and carbachol (see Fig. 5). Ryanodine is known as an agent that locks the Ca^{2+}-releasing channels of the ryanodine-sensitive Ca^{2+} store sites open, and that it depletes stored Ca^{2+} when applied with caffeine (Iino et al., 1988). As shown in Fig. 5, the caffeine-induced response was highly sensitive to ryanodine but the carbachol-induced response was not, suggesting that the Ca^{2+}-releasing channels involved in the responses to caffeine and carbachol are different. This supports the idea that caffeine and carbachol stimulate the release of Ca^{2+} from their specific Ca^{2+} stores.

Thapsigargin, an inhibitor of the ATP-dependent Ca^{2+} pump, abolished both I_{CAF} and I_{CCh} (not shown), and the $[Ca^{2+}]_i$ elevation evoked by caffeine and carbachol (Fig. 5C). These results suggest that the Ca^{2+} pumps that recharge the Ca^{2+} stores to maintain the responses to caffeine and carbachol have a similar thapsigargin sensitiv-

ity. All these data suggest that the caffeine- and carbachol-induced responses involve specific Ca^{2+} storage sites. Alternatively, caffeine and carbachol may act on the same store but carbachol may be a more efficient Ca^{2+}-releaser that can elicit responses under the Ca^{2+}-free, nicardipine- and ryanodine-treated conditions.

It is important to know whether both the Ca^{2+} storage sites involved in the responses to caffeine and carbachol are entirely independent of each other. Interestingly, we observed an apparently mutual interference between I_{CAF} and I_{CCh} when both stimulants were applied together (see Fig. 6A, B and D) in spite of their distinct $[Ca^{2+}]_o$ dependence and ryanodine sensitivity. The mutual interference between caffeine- and carbachol-induced responses was also observed for the $[Ca^{2+}]_i$ elevation elicited by both stimulants (Fig. 6C and E). These results suggest that the Ca^{2+} storage sites interact with each other. In the interaction between simultaneously evoked I_{CAF} and I_{CCh} and that between $[Ca^{2+}]_i$-elevations with both stimulants, a redistribution of Ca^{2+} between the caffeine- and carbachol-driven Ca^{2+} storage sites (Baro and Eisner, 1995). Alternatively, a mutual suppression of Ca^{2+} release from both types of Ca^{2+} store might occur in these phenomena. It is known that IP_3 receptor activity is facilitated at a proper $[Ca^{2+}]_i$, but inhibited at higher $[Ca^{2+}]_i$ (Iino and Endo, 1992). In the ryanodine-treated bladder smooth muscle cells, pretreatment with caffeine suppressed the carbachol-induced $[Ca^{2+}]_i$ elevation although caffeine did not elicit a $[Ca^{2+}]_i$ signal (see Fig. 5D). This suggests that the caffeine-induced suppression of the carbachol responses is not due to depletion of the carbachol-dependent Ca^{2+} stores. This is consistent with the finding that caffeine directly suppresses IP_3-induced Ca^{2+} release in rat hepatocytes (Missiaen et al., 1992b). The inhibition of the carbachol-induced contraction of bladder smooth muscle by caffeine (Fig. 1A, middle trace) may also include such a direct action of caffeine. The type of relationship between the two intracellular Ca^{2+} release mechanisms involved in the responses to both stimulants might be important in the characterization of the physiological function of distinct cell types.

References

Amédée, T., Benham, C.D., Bolton, T.B., Byrne, N.G., Large, W.A., 1990. Potassium, chloride and nonselective cation conductances opened by noradrenaline in rabbit ear artery cells. J. Physiol. 423, 551–568.

Baro, I., Eisner, D.A., 1992. The effects of thapsigargin on $[Ca^{2+}]_i$ in isolated rat mesenteric artery vascular smooth muscle cells. Pflüg. Arch. Eur. J. Physiol. 420, 115–117.

Baro, I., Eisner, D.A., 1995. Factors controlling changes in intracellular Ca^{2+} concentration produced by noradrenaline in rat mesenteric artery smooth muscle cells. J. Physiol. 482, 247–258.

Benham, C.D., Bolton, T.B., 1986. Spontaneous transient outward currents in single visceral and vascular smooth muscle cells of the rabbit. J. Physiol. 381, 385–406.

Berridge, M.J., Irvine, R.F., 1984. Inositol trisphosphate, a novel second messenger in cellular signal transduction. Nature 312, 315–321.

Blatter, L.A., Wier, W.G., 1992. Agonist-induced $[Ca^{2+}]_i$ waves and Ca^{2+}-induced Ca^{2+} release in mammalian vascular smooth muscle cells. Am. J. Physiol. 263, H576–586.

Bolton, T.B., Lim, S.P., 1989. Properties of calcium stores and transient outward currents in single smooth muscle cells of rabbit intestine. J. Physiol. 409, 385–401.

Carbone, E., Lux, H.D., 1987. Kinetics and selectivity of a low-voltage-activated calcium current in chick and rat sensory neurons. J. Physiol. 386, 547–570.

Fujii, T., Tokutomi, N., Hirata, A., Negi, A., Nishi, K., 1997. Cytoplasmic Ca^{2+} mobilization and Ca^{2+}-dependent membrane currents in dispersed bovine ciliary muscle cells. Curr. Eye Res. 16, 436–444.

Furuichi, T., Kohda, K., Miyawaki, A., Mikoshiba, K., 1994. Intracellular channels. Curr. Opin. Neurobiol. 4, 294–303.

Ganitkevich, V., Isenberg, G., 1990. Isolated guinea pig coronary smooth muscle cells, Acetylcholine induces hyperpolarization due to sarcoplasmic reticulum calcium release activating potassium channels. Circ. Res. 67, 525–528.

Giangiacomo, K.M., Garcia, M.L., McManus, O.B., 1992. Mechanism of iberiotoxin block of the large-conductance calcium-activated potassium channel from bovine aortic smooth muscle. Biochemistry 31, 6719–6727.

Horn, R., Marty, A., 1988. Muscarinic activation of ionic currents measured by a new whole-cell recording method. J. Gen. Physiol. 92, 145–159.

Iino, M., 1989. Calcium-induced calcium release mechanism in guinea pig *Taenia caeci*. J. Gen. Physiol. 94, 363–383.

Iino, M., 1991. Effects of adenine nucleotides on inositol 1,4,5-trisphosphate-induced calcium release in vascular smooth muscle cells. J. Gen. Physiol. 98, 681–698.

Iino, M., Endo, M., 1992. Calcium-dependent immediate feedback control of inositol 1,4,5-trisphosphate-induced Ca^{2+} release. Nature 360, 76–78.

Iino, M., Kobayashi, T., Endo, M., 1988. Use of ryanodine for functional removal of the calcium store in smooth muscle cells of the guinea pig. Biochem. Biophys. Res. Commun. 152, 417–422.

Iino, M., Yamazawa, T., Miyashita, Y., Endo, M., Kasai, H., 1993. Critical intracellular Ca^{2+} concentration for all-or-none Ca^{2+} spiking in single smooth muscle cells. EMBO J. 12, 5287–5291.

Inoue, R., Isenberg, G., 1990. Acetylcholine activates nonselective cation channels in guinea pig ileum through a G protein. Am. J. Physiol. 258, C1173–C1178.

Inoue, R., Kitamura, K., Kuriyama, H., 1985. Two Ca-dependent K-channel classified by the application of tetraethylammonium distribute to smooth muscle membranes of the rabbit portal vein. Pflüg. Arch. Eur. J. Physiol. 405, 173–179.

Kurachi, Y., Asano, Y., Takikawa, R., Sugimoto, T., 1989. Cardiac Ca current does not run down and is very sensitive to isoprenaline in the nystatin-method of whole cell recording. Naunyn-Schmiedeberg's Arch. Pharmacol. 340, 219–222.

Leijten, P.A.A., Van Breemen, C., 1984. The effects of caffeine on the noradrenaline-sensitive calcium store in rabbit aorta. J. Physiol. 357, 327–339.

Matsumoto, M., Nakagawa, T., Inoue, T., Nagata, E., Tanaka, K., Takano, H., Minowa, O., Kuno, J., Sakakibara, S., Yamada, M., Yoneshima, H., Miyawaki, A., Fukuuchi, Y., Furuichi, T., Okano, H., Mikoshiba, K., Noda, T., 1996. Ataxia and epileptic seizures in mice lacking type 1 inositol 1,4,5-trisphosphate receptor. Nature 379, 168–171.

Missiaen, L., De Smedt, H., Droogmans, G., Himpens, B., Casteels, R., 1992a. Calcium ion homeostasis in smooth muscle. Pharmacol. Ther. 56, 191–231.

Missiaen, L., Taylor, C.W., Berridge, M.J., 1992b. Luminal Ca^{2+} promoting spontaneous Ca^{2+} release from inositol trisphosphate-sensitive stores in rat hepatocytes. J. Physiol. 453, 623–640.

Murase, K., Randic, M., Shirasaki, T., Nakagawa, T., Akaike, N., 1990. Serotonin suppresses *N*-methyl-D-aspartate responses in acutely isolated spinal dorsal horn neurons of the rat. Brain Res. 525, 84–91.

Neering, I.R., McBurney, R.N., 1984. Role for microsomal Ca storage in mammalian neurones?. Nature 309, 158–160.

Ohta, T., Ito, S., Nakazato, Y., 1994. All-or-nothing responses to carbachol in single intestinal smooth muscle cells of rat. Br. J. Pharmacol. 112, 972–976.

Rousseau, E., Smith, J.S., Henderson, J.S., Meissner, G., 1986. Single channel and $^{45}Ca^{2+}$ flux measurements of the cardiac sarcoplasmic reticulum calcium channel. Biophys. J. 50, 1009–1014.

Somlyo, A.P., Somlyo, A.V., 1994. Signal transduction and regulation in smooth muscle. Nature 372, 231–236.

Somlyo, A.V., Bond, M., Somlyo, A.P., Scarpa, A., 1985. Inositol trisphosphate-induced calcium release and contraction in vascular smooth muscle. Proc. Natl. Acad. Sci. USA 82, 5231–5235.

Takeshima, H., 1993. Primary structure and expression from cDNAs of the ryanodine receptor. Ann. N.Y. Acad. Sci. 707, 165–177.

Takeshima, H., Iino, M., Takekura, H., Nishi, M., Kuno, J., Minowa, O., Takano, H., Noda, T., 1994. Excitation–contraction uncoupling and muscular degeneration in mice lacking functional skeletal muscle ryanodine-receptor gene. Nature 369, 556–559.

Takeshima, H., Ikemoto, T., Nishi, M., Nishiyama, N., Shimuta, M., Sugitani, Y., Kuno, J., Saito, I., Endo, M., Iino, M., Noda, T., 1996. Generation and characterization of mutant mice lacking ryanodine receptor type 3. J. Biol. Chem. 271, 19649–19652.

Tokutomi, N., Maeda, H., Tokutomi, Y., Sato, D., Sugita, M., Nishikawa, S., Nishikawa, S., Nakao, J., Imamura, T., Nishi, K., 1995. Rhythmic Cl^- current and physiological roles of the intestinal c-kit-positive cells. Pflüg. Arch. Eur. J. Physiol. 431, 169–177.

Yamazawa, T., Iino, M., Endo, M., 1992. Presence of functionally different compartments of the Ca^{2+} store in single intestinal smooth muscle cells. FEBS Lett. 301, 181–184.

Wakamori, M., Hidaka, H., Akaike, N., 1993. Hyperpolarizing muscarinic responses of freshly dissociated rat hippocampal CA1 neurons. J. Physiol. 463, 585–604.