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Presence of functionally different compartments of the Ca^{2+} store in single intestinal smooth muscle cells

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Studies in smooth muscle bundles have shown the presence of functionally different compartments of Ca^{2+} store, one (Sα) sensitive to both caffeine and inositol 1,4,5-trisphosphate (IP_3), and the other (Sβ) sensitive only to IP_3. Ca^{2+} release in isolated single smooth muscle cells from guinea pig taenia caeci was studied to see if both compartments exist within a cell. Responses to caffeine and carbachol were consistently observed but were abolished after treatment with ryanodine, while intracellular application of IP_3 induced Ca^{2+} release after the treatment, albeit smaller in size than control. Thus Sα and Sβ coexist in a single smooth muscle cell and agonist-induced Ca^{2+} release requires whole store to be loaded with Ca^{2+}.

Calcium storage; Caffeine; Inositol trisphosphate; Ryanodine; Carbachol

1. INTRODUCTION

Contractions of smooth muscle cells are regulated by the rise and fall of intracellular Ca^{2+} concentration, which is in turn controlled either by the Ca^{2+} influx through Ca^{2+} channels located on the surface membrane and/or by the Ca^{2+} release from intracellular Ca^{2+} stores [1]. In certain smooth muscle cells Ca^{2+} release from the intracellular Ca^{2+} stores is sufficiently large to induce maximal contraction [2]. Two Ca^{2+} release mechanisms have been found in smooth muscle Ca^{2+} store, i.e. Ca^{2+}-induced Ca^{2+} release (CICR), which is sensitive to caffeine [3], and inositol 1,4,5-trisphosphate (IP_3)-induced Ca^{2+} release (IICR) [4,5].

Our previous studies on skinned smooth muscle fiber bundles indicate that the Ca^{2+} store of smooth muscle cells consists of at least two compartments, one with both CICR and IICR mechanisms (Sα) and the other with only the IICR mechanism (Sβ) [6]. Therefore, only Sα is sensitive to caffeine. However, as the previous studies were carried out using multicellular preparations, it is not yet clear whether both Sα and Sβ exist in a single living cell or whether there are two types of cells with different Ca^{2+} stores. In the present study, to clarify the above question, Ca^{2+} release from enzymatically isolated intact single smooth muscle cells was examined.

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2. MATERIALS AND METHODS

2.1. *Cell preparation*

Suspensions of isolated smooth muscle cells were prepared by an enzyme treatment dispersion method [7] modified as follows: strips of taenia caeci were removed from male guinea pigs (250–300 g) exsanguinated to death, and were placed at 37°C for 30 min in a physiological salt solution (PSS) which contained (mM): NaCl 140; KCl 4; $CaCl_2$ 2; $MgCl_2$ 2; HEPES (*N*-2-hydroxyethylpiperazine-*N'*-ethanesulfonic acid)) 10; glucose, 10 (pH adjusted to 7.4 with NaOH), and then for 30 min in a Ca^{2+}-free (no $CaCl_2$) PSS. Each strip was cut into pieces 2–3 mm in length, and suspended in a low-Ca^{2+} (50 μM $CaCl_2$) PSS containing collagenase (1 mg/ml), trypsin inhibitor (1 mg/ml), and bovine serum albumin (5 mg/ml), for 30 min at 37°C. Following the enzyme treatment, single cells were dispersed by gentle pipetting and were kept at 4°C until use on the same day in a modified KB solution [13,14] (in mM): taurine 10; oxalic acid 10; L-glutamic acid 70; KCl 10; KH_2PO_4 10; glucose 11; HEPES, 10; Na_2ATP 0.5; pH adjusted to 7.35 with KOH.

2.2. *Experimental apparatus and protocol*

A suspension of single cells was poured into the well of a Perspex plate to let the cells sink and stick to the glass bottom. Floating cells were washed away by a flow of PSS which was kept running throughout the experiments. The cells left in the well were loaded with fura-2 in PSS containing 5 μM fura-2-acetoxymethyl ester [8] for 20 min at room temperature. In some experiments 0.1–0.2 μM cytochalasin D was treated in PSS for 5 min to suppress movements of the cells upon activation [9]. We could not observe any obvious effect of the cytochalasin D treatment on the Ca^{2+} transients in single cells.

The fluorescence intensity of fura-2-loaded single cells was measured with double wave length excitation at 340 and 380 nm alternating at 1,000 Hz using a fluorometer (CAM-230; Nihon Bunko Kogyo, Tokyo, Japan) attached to an epi-fluorescence in inverted microscope (TMD, Nikon, Tokyo, Japan). The fluorescence signals were electronically separated and low pass filtered at 10 Hz. Calibration of fura-2 signals against Ca^{2+} concentration (buffered with 10 mM EGTA, ethyleneglycol-bis[β-aminoethyl ether] *N,N,N',N'*-tetraacetic acid) was carried out using droplets of solutions with 4 μM fura-2 in silicone oil. The fluorescence intensity ratios in the cells were assumed to be smaller by 15% than in the calibrating solutions due to the viscosity effect [10].

Carbachol (CCh) and caffeine were applied to the cells using a 4-channel puffing pipette [11], which changed the solution surrounding the cells within ~500 ms. The tight-seal whole-cell configuration of patch-clamp technique [12] (EPC-7, List, Germany) was used to apply IP_3 intracellularly with simultaneous measurement of membrane currents at 0 mV holding potential. Patch pipettes had a resistance of 3–4 MΩ and contai .ed a solution of the following composition (mM): KCl 150; $MgMs_2$ (magnesium methanesulfonate) 0.1; Na_2ATP 5; EGTA 0.075; IP_3 0.1; HEPES 10 (pH adjusted to 7.2 with KOH). Experiments were carried out at room temperature (21–23°C).

3. RESULTS

3.1. *Responses to caffeine, carbachol and IP_3 of single isolated smooth muscle cells*

As shown in Fig. 1, which is a representative record of 7 cells, both 25 mM caffeine and 30 μM CCh applied for ~15 s in the Ca^{2+}-deprived (no $CaCl_2$ with 2 mM EGTA) PSS induced a transient increase in $[Ca^{2+}]_i$ of a single cell. Before each challenge the cell was left in PSS for 3 min to allow the Ca^{2+} store to take up Ca^{2+}, and $[Ca^{2+}]_o$ was reduced to zero 8 s before the drug application. The doses of caffeine and CCh were enough to obtain maximal responses, and the size of Ca^{2+} transients were the same at 5 mM caffeine or at 10 μM CCh. After the cell was shown to respond to the agonists repeatedly (a–d), the whole-cell patch clamp configuration by a pipette with 100 μM IP_3 was established to apply IP_3 intracellularly (Fig. 1e). There was a rapid increase in $[Ca^{2+}]_i$ and an outward current, which is likely to be a Ca^{2+}-activated K^+ current [15] because the current was absent if K^+ in the pipette solution was replaced with Cs^+ (not shown). The IP_3-induced Ca^{2+} release was smaller in peak size than the caffeine- or CCh-induced Ca^{2+} transients probably due to diffusion delay in the application of IP_3 through the patch pipette.

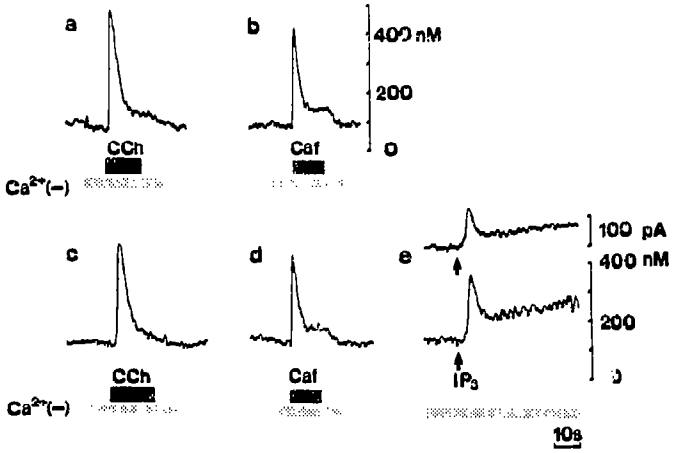

Fig. 1. Fluorescence ratio and current record obtained from a single smooth muscle cell. 30 μM CCh (a,c) or 25 mM caffeine (b,d) was applied in the absence of extracellular Ca^{2+} to induce intracellular Ca^{2+} release. Between each challenge, the cell was incubated in PSS for 3 min to reload the Ca^{2+} store. Ca^{2+} release and an outward current were observed upon establishment of whole-cell clamp to apply IP_3 intracellularly through a patch pipette (e).

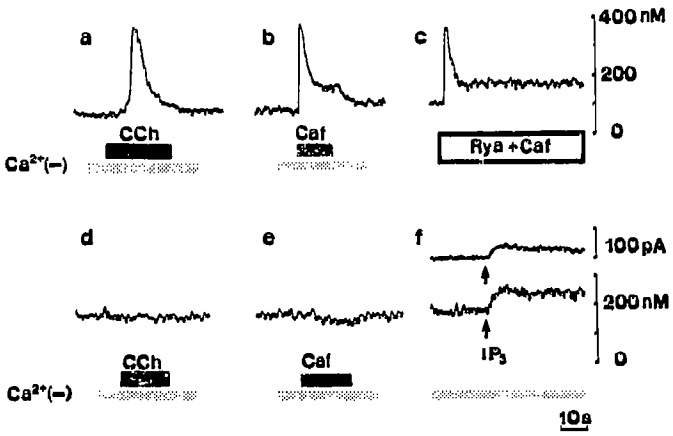

Fig. 2. The same protocol as for Fig. 1, except that 30 μM ryanodine was treated with 25 mM caffeine (c) after (a) and (b). This treatment irreversibly abolished the subsequent caffeine and CCh responses (d and e). However, IP_3 applied intracellularly still induced Ca^{2+} release (f).

If IP_3 was absent in the patch pipette, there was no change in the current or $[Ca^{2+}]_i$ in all the cells tested (n = 5), although increase in $[Ca^{2+}]_i$ were observed in these cells upon puffing of caffeine or CCh before the establishment of whole-cell clamp configuration.

3.2. *Ca^{2+} release following ryanodine treatment*

Ryanodine has been shown to deplete Sα by leaving the CICR channels in an open-locked state [6,16–18]. We, therefore, studied if intracellular application of IP_3 would induce Ca^{2+} release in ryanodine-treated single smooth cells to see if Sα and Sβ exist in the same cell. In Fig. 2, after the cell was confirmed to respond to CCh (a) and to caffeine (b), 30 μM ryanodine was applied for 100 s with 25 mM caffeine (c). Following the ryanodine treatment, applications of caffeine and CCh failed to induce Ca^{2+} release (d,e), in accordance with the previous results in smooth muscle bundles [6]. However, IP_3 applied intracellularly in the absence of extracellular Ca^{2+} still induced $[Ca^{2+}]_i$ rise and an outward current (f). The same results were obtained in all the 7 cells tested. In another set of experiments in which outward currents were suppressed by Cs^+ in the patch pipette, we also observed $[Ca^{2+}]_i$ rise upon intracellular application of IP_3 after the ryanodine treatment in all the cells (n = 11).

In Fig. 3 averaged peak sizes of the Ca^{2+} responses to CCh, caffeine and IP_3 were compared with or without the ryanodine treatment. There was no response for caffeine and CCh after the ryanodine treatment and the size of the IP_3-induced Ca^{2+} increase was reduced to about half of that without the ryanodine treatment.

3.3. *Ca^{2+} mobilized by carbachol and IP_3 after caffeine application*

The above results show that CCh-induced Ca^{2+} release is abolished when Sα has been depleted by the

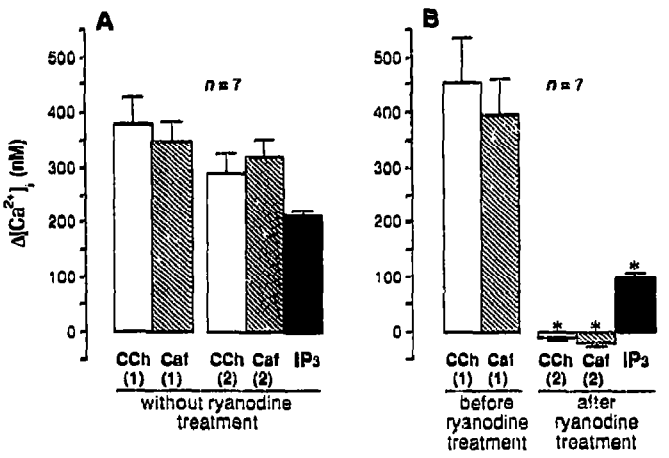

Fig. 3. Peak Ca^{2+} response sizes (peak Ca^{2+} concentration minus the value prior to the stimulus, $\Delta[Ca^{2+}]_i$) in response to caffeine, CCh and IP_3 (mean ± S.E.M.) with (B) or without (A) the ryanodine treatment. The experimental conditions were the same as in Figs. 1 and 2. Differences between the corresponding columns in A and B were statistically (unpaired t-test) significance ($P < 0.01$) where indicated by * or insignificant ($P > 0.3$) elsewhere. The negative values after the ryanodine treatment were due to slow decline in $[Ca^{2+}]_i$ in Ca^{2+}-deprived PSS.

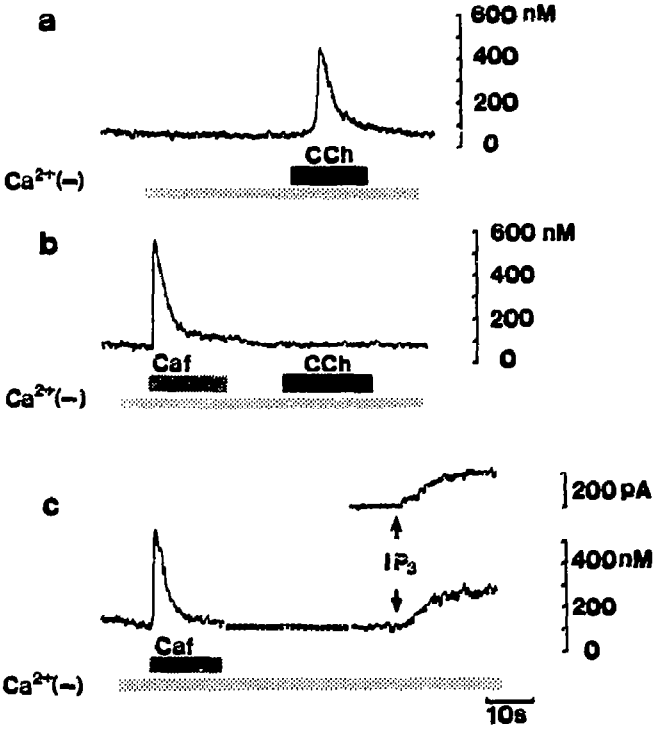

Fig. 4. Effects of caffeine pretreatment on the following CCh- or IP_3-induced Ca^{2+} release in a single smooth muscle cell. (a) Ca^{2+} release by 30 μM CCh after incubation in Ca^{2+}-deprived PSS for 40 s. (b) If 25 mM caffeine was applied in the absence of $[Ca^{2+}]_o$, the following application of 30 μM CCh failed to increase $[Ca^{2+}]_i$. (c) Intracellular application of IP_3 induced Ca^{2+} release and an outward current even after the application of 25 mM caffeine.

ryanodine treatment. We then examined CCh- and IP_3-induced Ca^{2+} release with caffeine pretreatment. Following 25 mM caffeine-induced Ca^{2+} release, 30 μM CCh failed to induce Ca^{2+} release (Fig. 4b), whereas without the caffeine pretreatment CCh did induce Ca^{2+} release in the same cell (Fig. 4a). However, intracellular application of IP_3 induced definite $[Ca^{2+}]_i$ increase after the caffeine application (c). The same results were obtained in 5 other cells. Thus CCh-induced Ca^{2+} release disappears after caffeine pretreatment, although a part of IP_3 sensitive store is left unreleased.

4. DISCUSSION

The present study shows that in single smooth muscle cells from guinea pig taenia caeci of which Sα has been depleted by pretreatment with ryanodine, intracellular application of IP_3 still increases $[Ca^{2+}]_i$. Therefore, it is clearly demonstrated that both Sα and Sβ are contained in a single intact smooth muscle cell. It is therefore important to assign these functions to intracellular organellas in future experiments. In cultured rat aortic smooth muscle cells, the presence of two types of cells has been reported [19], i.e. one sensitive only to caffeine and the other only to angiotensin II. This might be due to issue difference, but it seems possible that different population of cells appear due to cell culture because the responses to caffeine and angiotensin II changes during cell culture stage [20].

Heterogeneous compartments of intracellular Ca^{2+} store may be present not only in smooth muscle cells but in nerve cells. A histochemical study has revealed that CICR channels (ryanodine receptors) and IP_3 receptors coexist in the same cell with different distribution patterns in Purkinje cells of avian cerebellum, and that only the IP_3 receptors are present in dendritic spines [21]. These morphological studies suggest the presence of functionally different compartments in nerve cells, although direct evidence is still wanting.

Agonists such as carbachol (CCh) may evoke Ca^{2+} release by the formation of IP_3 [4,5,22]. Previous reports on intact smooth muscle bundles show that the ryanodine treatment to deplete caffeine-sensitive compartments (Sα) results in abolition or extensive reduction of agonist-induced Ca^{2+} release [6,16–18,23]. The present study demonstrates this result at the level of individual cells and CCh-induced contraction was never observed after Ca^{2+} in Sα had been released either by previous treatment with caffeine or with ryanodine in single cells, although a part of IP_3-sensitive compartment was left loaded with Ca^{2+}. One of the ways to explain this puzzling result is to suppose that CCh preferentially releases Ca^{2+} from Sα (targeting mechanism), although the reason why IP_3-sensitive Sβ may not be utilized requires elucidation. It may be that Sβ is located deep inside the cell, and IP_3 formed at the cell membrane can hardly reach these sites because of the rapid metabolism by cytosolic enzymes [23,24], i.e. IP_3 phosphatase and

IP_3 kinase. It is also conceivable that the coexisting CICR channels in Sα accelerate Ca^{2+} release. However, none of these possibilities has been critically tested. Another possibility is impairment of positive feedback control of IICR (feedback control mechanism). Because the rate of IICR depends on the Ca^{2+} concentration [25,26], a rise in Ca^{2+} concentration due to Ca^{2+} release is expected to feedback to the Ca^{2+} release rate. If the amount of Ca^{2+} in the store declines, then the Ca^{2+} release may take place less efficiency because the positive feedback loop is weakened.

Our results, showing that agonist-induced Ca^{2+} release in smooth muscle cells requires both Sα and Sβ to be loaded with Ca^{2+}, indicate either the targeting mechanism or the feedback control mechanism or a still unspecified mechanism is involved in the intracellular signal transduction in smooth muscle cells. It will be important to elucidate this mechanism in future studies.

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