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Caffeine and carbachol act on common Ca^{2+} stores to release Ca^{2+} in guinea-pig ileal smooth muscle

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Abstract

To characterize intracellular Ca^{2+} stores, the Ca^{2+}-releasing effects of caffeine, carbachol and inositol 1,4,5-trisphosphate (IP_3) were compared by measuring the drug-induced tension development in β-escin-skinned longitudinal smooth muscle of guinea-pig ileum. Caffeine (20 mM), carbachol (10 or 100 μM) or IP_3 (40 μM), applied after loading Ca^{2+} within intracellular stores, produced a transient rise in tension in a Ca^{2+}-free solution. This change in tension occurred in response to release of Ca^{2+} from the stores. The effect of either caffeine or carbachol was markedly reduced or abolished after preceding application of the other drug. IP_3 was without effect when applied subsequently to caffeine. The effects of carbachol and IP_3 were abolished after combined treatment with ryanodine (30 μM) and caffeine (20 mM) which causes functional removal of caffeine-releasable Ca^{2+} stores, but not after combined treatment with ryanodine (30 μM) and carbachol (10 μM). The results suggest that caffeine, carbachol and IP_3 all act on common Ca^{2+} stores to release Ca^{2+}.

Keywords: Ca^{2+} store; Caffeine; Carbachol; Inositol 1,4,5-trisphosphate; Ryanodine; Smooth muscle, intestinal

1. Introduction

It is well known that two types of Ca^{2+} release mechanism operate in intracellular Ca^{2+} stores of smooth muscle; one is the inositol 1,4,5-trisphosphate (IP_3)-induced Ca^{2+} release mechanism (Suematsu et al., 1984; Somlyo et al., 1985) and the other is the Ca^{2+}-induced Ca^{2+} release mechanism (Saida, 1982; Iino, 1989). The sensitivity of the latter type of Ca^{2+} release mechanism to Ca^{2+} is increased by caffeine (Iino, 1989). Iino et al. (1988) suggested that two types of Ca^{2+} stores, one ($S\alpha$) having both Ca^{2+}-induced Ca^{2+} release mechanism and IP_3-induced Ca^{2+} release mechanism and being capable of releasing Ca^{2+} in response to both caffeine and IP_3, and the other ($S\beta$) having the IP_3-induced Ca^{2+} release mechanism alone and being capable of releasing Ca^{2+} in response to IP_3, exist in various smooth muscles with different relative sizes of these ($S\alpha$ and $S\beta$). The studies leading to this notion were undertaken with saponin-skinned smooth muscle strips (Iino, 1987; Iino et al., 1988).

In single intact smooth muscle cells from rabbit jejunum, carbachol or microinjected IP_3, activators of IP_3-induced Ca^{2+} release mechanism, failed to elevate the intracellular Ca^{2+} concentration after depletion of caffeine-releasable Ca^{2+} stores, as judged by measuring Ca^{2+}-activated K^+ current (Komori and Bolton, 1990, 1991). Similar results were obtained in single cells from guinea-pig ileum (Komori et al., 1992). These experiments suggest no existence of $S\beta$ at least in these smooth muscle cells. However, cells used for such experiments with single cells were limited in number so that a possible existence of cells with $S\beta$ in addition to $S\alpha$ or $S\beta$ alone is still not eliminated.

β-Escin, unlike saponin, permeabilizes the cell membrane leaving receptor-coupled signal transduction systems intact (Kobayashi et al., 1989; Kitazawa and Somlyo, 1990). Therefore, smooth muscles permeabilized with β-escin respond to a variety of receptor agonists such as carbachol, not to mention caffeine and IP_3, and serve for various studies on intracellular Ca^{2+} mobilization (e.g., Kobayashi et al., 1989; Uyama et al., 1992; Fukami et al., 1993; Nagasaki et al., 1994).

The present study was designed to analyse intracellular Ca^{2+} stores mobilized by caffeine, carbachol and

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IP_3 in β-escin-skinned longitudinal smooth muscle of guinea-pig ileum in an attempt to clarify the nature of Ca^{2+} stores and mechanisms of Ca^{2+}-induced Ca^{2+} release and IP_3-induced Ca^{2+} release. Ca^{2+} release from store sites was detected by measuring tension development, the magnitude of which correlates with an increase in cytosolic free Ca^{2+} concentration. For functional removal of caffeine-releasable Ca^{2+} stores, ryanodine was applied simultaneously with caffeine (Iino et al., 1988; Yamazawa et al., 1992).

2. Materials and methods

Male guinea-pigs (350–450 g) were exsanguinated. A small muscle strip, 4–6 mm in length and 0.2–0.3 mm in width, was prepared from the longitudinal muscle layer of the ileum, was mounted horizontally in a 0.3-ml organ bath and had one end fixed on the rubber bottom of the bath and the other attached to a thin lever of an isometric force transducer, as described elsewhere (Fukami et al., 1993). The organ bath was filled with a physiological salt solution (PSS; composition given below) kept at 23°C and the muscle strip was equilibrated under a tension of 150–180 mg for 30–60 min. Then, skinning of the muscle cells was performed by incubating the muscle strip with β-escin (40–60 μM) in a Ca^{2+}-containing solution (pCa 6; composition given below) for approximately 30 min until the gradual rise in tension reached a steady level. After skinning, the muscle strip was bathed in a relaxing solution containing 4 mM EGTA (RI solution, composition given below).

Intracellular Ca^{2+} stores of the skinned ileal muscle were loaded with Ca^{2+} by replacing the bath medium (RI solution) with Ca^{2+}-containing solution (pCa 6) for 10 min. Caffeine, carbachol or IP_3 was applied for 1–1.5 min by replacing the RI solution with another relaxing solution (RII solution, composition given below) to which these drugs were added. Since the Ca^{2+}-releasing effect mediated by G-protein-coupled receptors is usually unstable in chemically skinned smooth muscle, GTP (50 μM) was allowed to be present during application of carbachol in order to replenish the loss of endogeneous GTP (Kitazawa et al., 1989; Kobayashi et al., 1989).

The PSS was a Hepes-buffered, modified Krebs solution containing (mM): NaCl, 126; KCl, 6; $CaCl_2$, 2; $MgCl_2$, 1.2; glucose, 14; Hepes, 10.5 (pH adjusted to 7.2 with NaOH at room temperature). The composition of the relaxing solution was (mM): K propionate, 130; $MgCl_2$, 4; Na_2ATP, 4; Tris-maleate, 20; creatine phosphate, 10; creatine phosphokinase (3.3 units/ml); EGTA, 4 (for RI solution) or 0.05 (for RII solution) (pH adjusted to 6.8 with KOH). A Ca^{2+}-containing solution (pCa 6) used for Ca^{2+}-loading and muscle-skinning was prepared by using Ca^{2+}-EGTA buffer with 2 mM EGTA and 1 mM Ca^{2+} assuming an apparent binding constant of 10^6/M for the Ca^{2+}-EGTA complex at pH 6.8 (Nishimura et al., 1988).

Drugs used were guanosine triphosphate (GTP; Sigma), inositol 1,4,5-trisphosphate (IP_3; Sigma), carbachol chloride (Tokyo Kasei), caffeine (Wako) and ryanodine (Wako). All other reagents were of the highest grade commercially available.

3. Results

3.1. General observations on caffeine- and carbachol-induced tension development due to release of Ca^{2+} stores

During Ca^{2+}-loading (see Methods), a rise in tension occurred and reached a peak within 3 min. The peak tension remained almost unchanged or declined gradually by less than 30%, as described previously (Fukami et al., 1993).

Caffeine (1–40 mM), applied 4 min after reintroduction of a relaxing solution (RI solution) following Ca^{2+}-loading, produced a transient rise in tension due to release of stored Ca^{2+}. The tension responses to caffeine reached a peak within 0.5–1 min and then declined to close to their level before caffeine application in its continued presence. The concentration-dependence of the Ca^{2+}-releasing effect was determined by means of a double-application protocol. Caffeine was applied first at a concentration of 2, 5, 10 or 20 mM and 2–3 min later at 40 mM in the continued Ca^{2+}-free environment after Ca^{2+}-loading (see Fig. 1A). The idea is that the magnitude of the second (40 mM) caffeine-induced tension development reflects the remaining amount of stored Ca^{2+} in caffeine-releasable stores. Thus, the magnitude of the ratio of the second tension response to the sum of the first and second tension responses is inversely proportional to the Ca^{2+}-releasing effect of caffeine applied first. Fig. 1B shows plots of the ratio (relative amplitude) against the concentration of caffeine. Caffeine released Ca^{2+} from stores in a concentration-dependent manner, and the concentration of 20 mM was high enough to deplete the stores almost completely. When a pair of Ca^{2+}-loading and subsequent application of 20 mM caffeine was repeated three times at an interval of 25–30 min, the magnitude of the second and third tension responses was reduced to $91 \pm 6\%$ and $80 \pm 4\%$ ($n = 8$), respectively (Fig. 1C; a time-dependent rundown).

Carbachol (0.1–100 μM), applied in the same way as used for caffeine, elicited a transient rise in tension, but the tension response was reproducible only when the drug was applied in the presence of 50 μM GTP (see Methods). This suggests some loss of endogeneous GTP (Kitazawa et al., 1989; Kobayashi et al., 1989).

GTP itself affected neither basal tension nor the caffeine response. Hereafter, carbachol was always applied in the presence of GTP (50 μM). The concentration dependence of the Ca^{2+}-releasing effect of carbachol was determined in the same way as used for caffeine. Carbachol was applied first at a concentration of 0.1, 1 or 10 μM and then at 100 μM (see Fig. 2A). Fig. 2B shows a similar plot of the second carbachol-induced tension development against the concentration of the drug applied first to that for caffeine (Fig. 1B). In the case of carbachol, the concentration of 10 μM was high enough to cause almost complete depletion of Ca^{2+} from the stores. A time-dependent rundown of tension responses to 10 μM carbachol is illustrated in Fig. 2C. The magnitudes of the second and third tension responses were reduced to 89 ± 4% and 79 ± 4% (n = 9), respectively.

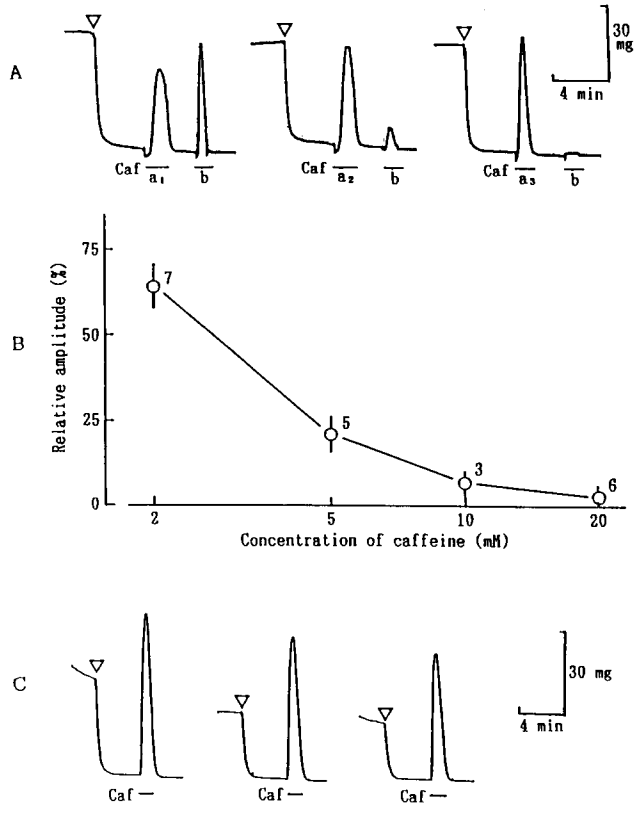

Fig. 1. Tension responses to repeated application of caffeine (Caf) in β-escin-skinned longitudinal smooth muscle of guinea-pig ileum. After skinning, the muscle was placed in a Ca^{2+}-containing solution at pCa 6 for 10 min for loading Ca^{2+} within intracellular stores (▽, the end of Ca^{2+} loading) and then in a relaxing solution containing 4 mM EGTA and no added Ca^{2+} for 4 min. Caf was applied for 1–1.5 min as indicated by bars. Another relaxing solution containing 0.05 mM EGTA and no added Ca^{2+} was used during the application of Caf. (A) A pair of tension responses to Caf applied first at a concentration of 2 mM (a_1; left panel), 5 mM (a_2; middle panel) or 20 mM (a_3; right panel) and then at 40 mM (b). (B) Plot of the ratio of the amplitude (%) of the second Caf (40 mM) response to the sum of the first and second Caf responses against the concentration of Caf applied first. Each point represents the mean ± S.E.M. of the measurements indicated by the attached numbers. (C) Tension responses to 20 mM Caf in three successive trials with one preparation. The period between two successive panels is 16 min.

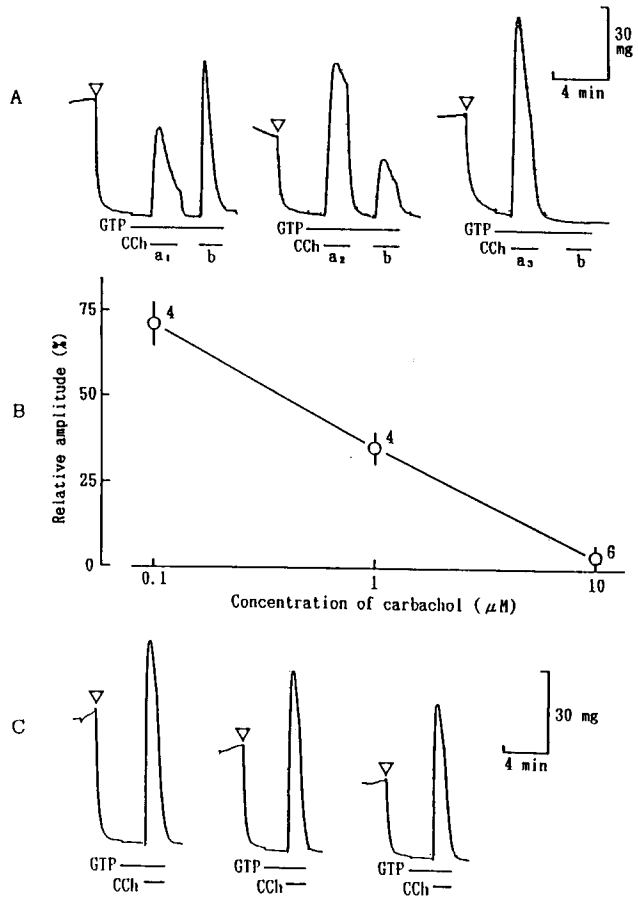

Fig. 2. Tension responses to repeated application of carbachol (CCh). The same protocol for exposure to the different solutions as in Fig. 1 was used, but CCh was always applied in the presence of 50 μM GTP as indicated by bars attached to GTP. (A) A pair of tension responses to CCh applied first at a concentration of 0.1 μM (a_1; left panel), 1 μM (a_2; middle panel) or 10 μM (a_3; right panel) and then at a given concentration of 100 μM (b). (B) Plot of the amplitude (%) of the second CCh (100 μM) response relative to the sum of the first and second CCh responses against the concentration of CCh applied first. Each point represents the mean ± S.E.M of the measurements indicated by attached numbers. (C) Tension responses to 10 μM CCh in three successive trials with one preparation. The period between two successive panels is 16 min.

The tension response to 10 μM carbachol was generally greater and slower to decline from the peak, compared with that to 20 mM caffeine. The difference between the carbachol and caffeine responses cannot be explained simply by differences in the amount of released Ca^{2+}. It could have resulted from sensitization of contractile elements to Ca^{2+} by carbachol (Kitazawa and Somlyo, 1990) and multiple actions of caffeine such as decrease in Ca^{2+} sensitivity of contractile elements and phosphodiesterase inhibition (Sato et al., 1988).

3.2. Interaction between the caffeine- and carbachol-induced tension developments

To characterize Ca^{2+} stores responsible for the Ca^{2+}-releasing effects of caffeine and carbachol, the availability of Ca^{2+} stores was tested by means of a second double-application protocol. Application of either 20 mM caffeine or 10 μM carbachol was followed at a 2 to 3-min interval by application of the other drug in the continued Ca^{2+}-free environment after Ca^{2+} loading, and this was repeated with a different order of applications to one preparation.

Application of carbachol subsequent to caffeine was without effect in three out of nine preparations but produced a rise in tension in the remainder ($28 \pm 9\%$ of that produced by application of carbachol alone, $n = 6$) (Fig. 3). When carbachol was applied first and caffeine was applied next, responses to caffeine were extremely small or absent in six out of the nine preparations (Fig. 3A,B). In the three remaining, caffeine produced a rise in tension with a size of $23 \pm 3\%$ ($n = 3$) of the peak tension produced by application of caffeine alone (Fig. 3). The finding that the Ca^{2+}-releasing effects of caffeine and carbachol were saturable (not additive with each other) suggests that the greater parts of the caffeine- and carbachol-releasable Ca^{2+} stores overlap functionally.

3.3. Effects of ryanodine on the caffeine- and carbachol-induced tension developments

It has been shown that ryanodine, if applied with caffeine, locks Ca^{2+}-induced Ca^{2+} release channels in an open state, and thus Ca^{2+} stores with these channels (i.e. caffeine-releasable Ca^{2+} stores) lose their capacity to retain Ca^{2+} inside (Iino et al., 1988). Therefore, the effects of ryanodine on the caffeine- and carbachol-induced tension developments were tested to see whether carbachol and caffeine share common Ca^{2+} stores from which to release Ca^{2+}. First, the control response to 20 mM caffeine or 10 μM carbachol was obtained 4 min after reintroduction of the RI solution following Ca^{2+} loading in one muscle preparation (Fig. 4A,B, left). Second, the muscle preparation was exposed to 20 mM caffeine for about 1 min in the presence of 30 μM ryanodine, applied 2 min before, in RI solution following Ca^{2+} loading (Fig. 4A,B, middle). Thereafter, both ryanodine and caffeine were washed away by replacing the bath medium with fresh RI solution. Third, the test in the first trial was repeated with the same muscle preparation (Fig. 4A,B, right). The simultaneous treatment with ryanodine and caffeine resulted in abolition of tension development in response to 10 μM carbachol as well as 20 mM caffeine. In four and three other preparations similar results were obtained for carbachol and caffeine, respectively. The average changes in tension responses are shown in Fig. 6. Even if carbachol was used at 100 μM, it had little effect on tension ($n = 3$, data not shown). The simultaneous treatment with ryanodine and caffeine produced no noticeable effect on tension development induced during subsequent Ca^{2+} loading, as shown in Fig. 4. Further, ryanodine itself did not interfere with activation of muscarinic receptors by carbachol leading to a massive release of Ca^{2+} stores (see below).

The results suggest that the ryanodine treatment (exposure to caffeine in the presence of ryanodine) does not allow the Ca^{2+} stores to be refilled with Ca^{2+} during subsequent Ca^{2+} loading and results in their functional removal. The abolition of the tension responses to carbachol as well as to caffeine by ryanodine may reflect the complete release of all releasable Ca^{2+} stores, in other words, carbachol-releasable Ca^{2+} stores

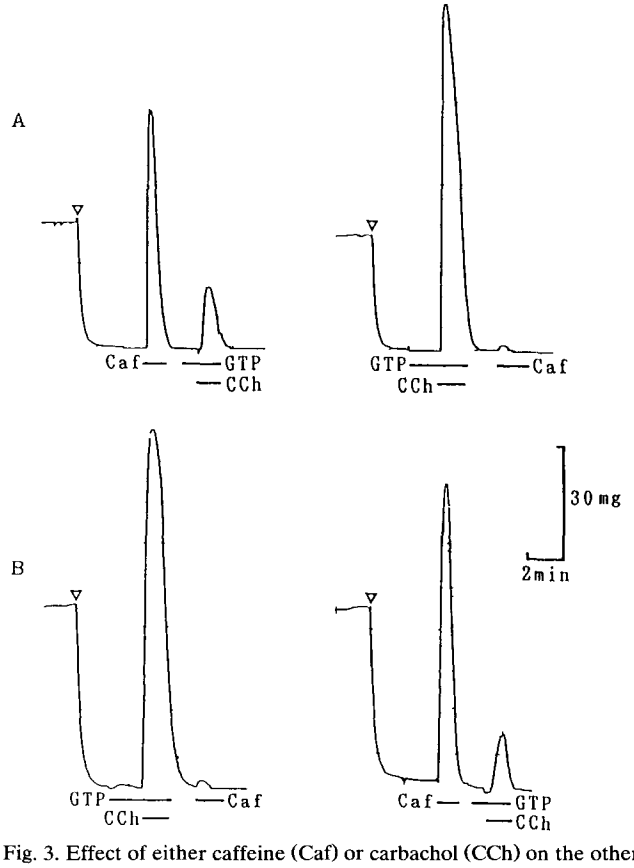

Fig. 3. Effect of either caffeine (Caf) or carbachol (CCh) on the other drug-induced tension development. The same protocol for exposure to the different solutions as in Fig. 1 was used. A pair of applications of 20 mM Caf and 10 μM CCh at a 2 to 3-min interval was repeated with changed application order in one preparation. (A) A pair of tension responses to Caf and to CCh (left panel) and those to CCh and to Caf (right panel). (B) A pair of tension responses to CCh and to Caf (left panel) and those to Caf and to CCh (right panel). The period between the left and right panels in (A) and (B) is 16 min. Note a marked inhibition of each drug-induced tension development by the other.

may be indistinguishable from caffeine-releasable Ca^{2+} stores.

When 10 μM carbachol, instead of 20 mM caffeine, was present during treatment with ryanodine, the following caffeine- and carbachol-induced tension developments remained almost unchanged (Fig. 5A and C). The peak tension was $94 \pm 8\%$ ($n = 6$) of the control for the caffeine response and $83 \pm 3\%$ ($n = 2$) for the carbachol response (see Fig. 6). The mean values were not significantly different from the corresponding values obtained by repeated application of either drug alone (Fig. 6) suggesting that the reduction may imply solely some rundown in tension responses to repeated applications of caffeine or carbachol. When 20 mM caffeine in addition to 10 μM carbachol was present during treatment with ryanodine, the caffeine-induced tension development was abolished ($n = 2$) (Fig. 5B and Fig. 6). The results indicate that ryanodine, when applied with carbachol, affects neither caffeine- nor carbachol-releasable Ca^{2+} stores.

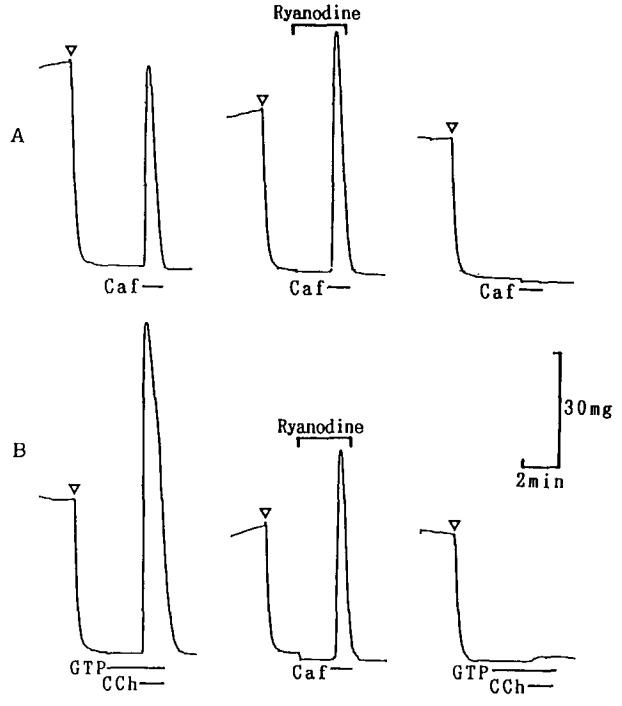

Fig. 4. Effect of combined treatment with ryanodine and caffeine (Caf) on Caf- and carbachol (CCh)-induced tension development. The same protocol for exposure to the different solutions as in Fig. 1 was used. The combined treatment was that 30 μM ryanodine was applied 2 min after Ca^{2+} loading, 2 min later 20 mM Caf was additively applied for 1 min and then both drugs were removed (see the middle panels). (A) Tension responses to 20 mM Caf before (left) and after (right) the combined treatment. (B) Tension responses to 10 μM CCh before (left) and after (right) the combined treatment. The period between the two successive panels is 16 min. Note abolition of both Caf- and CCh-induced tension developments after the combined treatment.

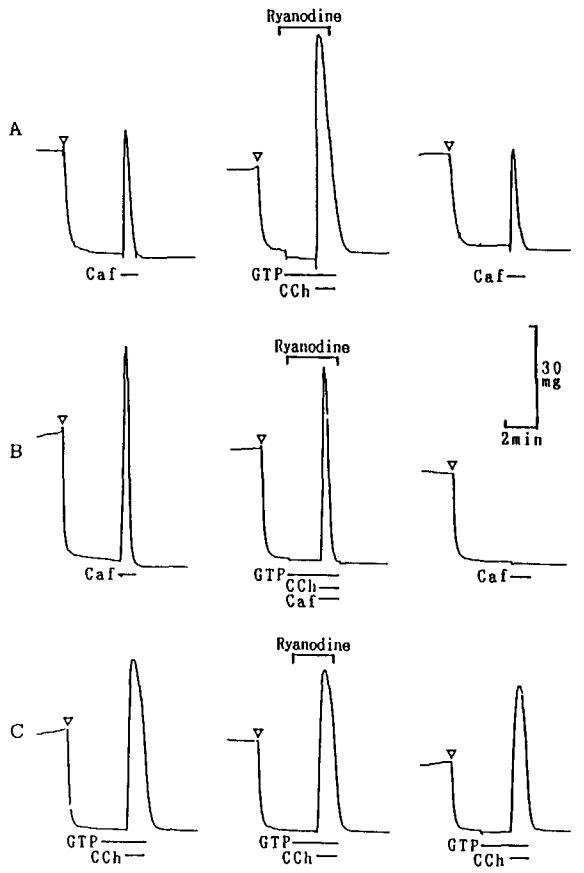

Fig. 5. Effect of combined treatment with ryanodine and carbachol (CCh) on caffeine (Caf)- and CCh-induced tension development. The same protocol for the different solutions as in Fig. 1 was used. The combined treatment was that 30 μM ryanodine was applied 2 min after Ca^{2+} loading, 2 min later 10 μM CCh was additively applied for 1 min and then both drugs were removed (see the middle panels in A and C). For the combined treatment in (B), 20 mM Caf was applied together with 10 μM CCh. (A),(B) Tension responses to 20 mM Caf before (left) and after (right) the combined treatment without and with 20 mM Caf, respectively. (C) Tension responses to 10 μM CCh before (left) and after (right) the combined treatment. Note that the combined treatment with ryanodine and CCh did not show any appreciable effect on either Caf- or CCh-induced tension development.

3.4. IP_3-induced tension development due to release of Ca^{2+} stores

Iino et al. (1988) have reported that, after depletion of caffeine-releasable Ca^{2+} stores, exogenously applied IP_3 still induces release of Ca^{2+} in saponin-skinned taenia caeci of the guinea-pig. Whether or not this is the case in β-escin-skinned ileal muscle was tested. First, the Ca^{2+}-releasing effects of 40 μM IP_3 (the maximum concentration used in the present work) and 20 mM caffeine were compared using the double-application protocol described in section 3.2. When IP_3 was first applied and then caffeine, tension responses to caffeine were obtained with mean size of $39 \pm 10\%$

($n = 4$) of that of the tension response to caffeine alone. When the order of application was reversed, IP_3 produced no tension response in the four preparations tested (data not shown).

Second, after combined treatment with ryanodine and caffeine, as described above, the effect of IP_3 on tension was tested. As shown in Fig. 7A, IP_3 (40 μM) induced no tension development in the four preparations tested. On the other hand, when application of caffeine alone, which allows Ca^{2+} stores to be refilled after its washout, was made instead of the ryanodine treatment, 40 μM IP_3 produced a tension development the peak of which was $71 \pm 4\%$ ($n = 4$) of that in response to the first application of IP_3 (Fig. 7B).

From the results, exogenously applied IP_3, like endogenous IP_3 resulting from stimulation of muscarinic receptors, seems unlikely to act on Ca^{2+} stores other than caffeine-releasable stores to release Ca^{2+}.

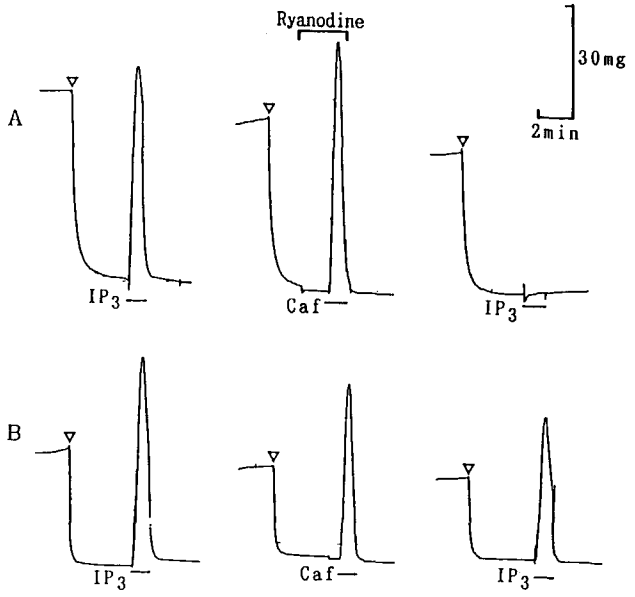

Fig. 7. Effect of combined treatment with ryanodine and caffeine (Caf) on IP_3-induced tension development. The same protocol for exposure to the different solutions as in Fig. 1 was used. The combined treatment (see the middle panel in A) was carried out in the same way as described in Fig. 4. (A) Tension responses to 40 μM IP_3 before (left) and after (right) the combined treatment. (B) Tension responses to 40 μM IP_3 before (left) and after (right) treatment with 20 mM Caf alone (middle). Note abolition of the IP_3-induced tension development after the combined treatment with ryanodine and Caf.

4. Discussion

The present experiments demonstrate that β-escin-skinned smooth muscle of guinea-pig ileum responds to carbachol as well as IP_3 and caffeine by tension development resulting from release of Ca^{2+} from its store sites, and that the tension response to carbachol or caffeine is reproducible with only a small time-dependent rundown (Fig. 1C and Fig. 2C). The muscle preparation has been shown to produce a detectable rise in tension when exposed to 320 nM of Ca^{2+} (Fukami et al., 1993). The properties of the muscle preparation allowed us to perform experiments to elucidate characteristics of Ca^{2+} stores.

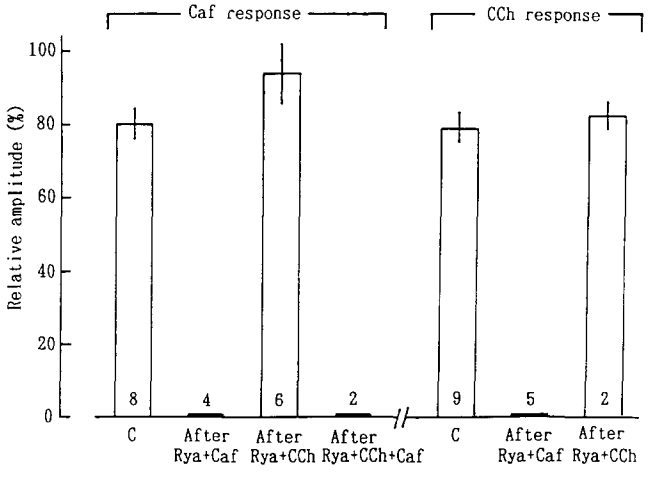

Fig. 6. Summary of the effects of treatment with ryanodine on caffeine (Caf)- and carbachol (CCh)-induced tension development. Experimental protocols were the same as described in Figs. 4 and 5. Ordinate; amplitudes (%) of the 20 mM Caf- or 10 μM CCh-tension response after 30 μM ryanodine treatment combined with 20 mM Caf (after Rya + Caf), 10 μM CCh (after Rya + CCh) or 10 μM CCh and 20 mM Caf (after Rya + CCh + Caf) relative to that before the ryanodine treatment, and of the third, relative to the first tension response to repeated applications of either 20 mM Caf or 10 μM CCh without any ryanodine treatment (see in Figs. 1C and 2C) (C). Each column represents the mean ± S.E.M. of the measurements indicated by attached numbers.

Carbachol, applied subsequently to caffeine, elicited a sizable rise in tension (see Fig. 3). This may be taken as evidence for a possible existence of Ca^{2+} stores releasable with IP_3 but not with caffeine ($S\beta$). However, the failure of carbachol to induce tension development after combined treatment with ryanodine and caffeine, which caused selectively functional removal of caffeine-releasable Ca^{2+} stores, strongly suggests no existence of $S\beta$. The apparently contradictory results are due to the concentrations of carbachol and caffeine which were not supra- but near maximal for their activities to release Ca^{2+} and critical levels to produce reproducible responses.

The present results provide evidence that intracellular Ca^{2+} stores of the longitudinal smooth muscle of guinea-pig ileum have both Ca^{2+}-induced and IP_3-induced Ca^{2+} release mechanisms and show no heterogeneity in terms of functional compartments. This is corroborated by results of our previous study in which the Ca^{2+}-activated K^+ current responses to caffeine and carbachol were investigated to characterize intra-

cellular Ca^{2+} stores in single isolated muscle cells from guinea-pig ileum (Komori et al., 1992). However, this is inconsistent with the notion derived from the measurements of Ca^{2+} in addition to tension that Sα, Ca^{2+} stores with both Ca^{2+} release mechanisms, and Sβ, Ca^{2+} stores with IP_3-induced Ca^{2+} release mechanism alone, exist in vascular and non-vascular smooth muscles (Iino, 1987; Iino et al., 1988; Yamamoto et al., 1991). The most acceptable explanation for the present results is that the ratio of Sα and Sβ may vary among smooth muscles and the size of Sβ is very small or negligible in the guinea-pig ileal muscle.

It is worth noting some characteristics of Sβ which has been proposed to exist in guinea-pig taenia caeci cells (Iino, 1987; Iino et al., 1988; Yamazawa et al., 1992). First, this compartment can release Ca^{2+} in response to exogenous IP_3 but not to endogenous IP_3 which has been well accepted as involved in agonist-induced Ca^{2+} release (Kobayashi et al., 1989). As explanation of such a peculiar property of Sβ, they raised the possibility that Sβ is located deep inside a smooth muscle cell and that IP_3 formed by receptor agonists in the plasma membrane hardly reaches Sβ. However, this is less likely because photolysis of caged IP_3 preloaded inside rabbit jejunal cells produced no response after exposure of the cells to carbachol to deplete Ca^{2+} (Komori and Bolton, 1991). Second, the Ca^{2+} release from Sβ, unlike that from Sα, occurs slowly and persists for a long time (Yamazawa et al., 1992); infusion of IP_3 (100 μM) from the patch pipette to a single cell interior, which had been treated with caffeine or ryanodine, produced a slowly developed and sustained rise in cystosolic Ca^{2+} level which was closely followed by a Ca^{2+}-activated K^+ current. Their observation might not be related to release of Ca^{2+} from store sites mediated by IP_3, but rather to operation of an unidentified process triggered by membrane rupture itself (Komori and Bolton, 1991). Further, the effective concentration of IP_3 to release stored Ca^{2+} is 1–2 μM (Suematsu et al., 1984; Yamamoto and van Breemen, 1985; Somlyo et al., 1992), which can be reached within a few seconds after break-through of the patch membrane when a patch pipette containing 100 μM IP_3 is used (Komori and Bolton, 1991). If so, the application of IP_3 is expected to bring about a rapid release of all IP_3-releasable Ca^{2+} stores. Under these circumstances, further studies need to be undertaken for exploration of the relevance of the existence of Sβ to smooth muscle physiology.

An interesting finding was that ryanodine failed to remove the Ca^{2+}-storage function of Ca^{2+} stores if it was applied with carbachol which exerted a near maximal effect in releasing Ca^{2+}. This, taken together with the result obtained with combined treatment with ryanodine and caffeine, suggests that ryanodine requires activation of the Ca^{2+}-induced Ca^{2+} release mechanism to act. The present findings support the mode of action of ryanodine proposed by Iino et al. (1988) that this drug acts on Ca^{2+}-induced Ca^{2+} release channels when they are open (due to caffeine application), and keeps them in an open state. Further, the failure of the combined treatment with ryanodine and carbachol to remove the Ca^{2+}-storage function implies that Ca^{2+} released by carbachol does not serve for further release of Ca^{2+} through activation of the Ca^{2+}-induced Ca^{2+} release mechanism. A simple explanation for this is that the Ca^{2+} concentration increased by carbachol is too low to activate the Ca^{2+} release mechanism. Iino (1989) reported that intracellular Ca^{2+} concentrations higher than 1 μM are required for activation of the Ca^{2+}-induced Ca^{2+} release mechanism in smooth muscle. In fact, carbachol, at the same concentration as used for the present study, produces an elevation of intracellular Ca^{2+} concentration up to 800 nM in single guinea-pig ileal cells (as measured with a fura-2 method; Kohda, Unno, Komori and Ohashi, unpublished data). However, immediately after activation of the IP_3-induced Ca^{2+} release mechanism it is possible that Ca^{2+} concentration in the vicinity of Ca^{2+} stores reaches a level higher than 800 nM. If so, another explanation is necessary for the inability of Ca^{2+} released by carbachol to activate Ca^{2+}-induced Ca^{2+} release channels. A possibility is that populations of channels responsible for IP_3- and Ca^{2+}-induced Ca^{2+} release are located at a certain interval from each other on the membrane of Ca^{2+} stores. The distance between them would serve as a structural barrier to access of Ca^{2+} released through IP_3-induced Ca^{2+} release channels to Ca^{2+}-induced Ca^{2+} release channels. Another possibility is that proteins such as calmodulin, which exhibit a high affinity toward Ca^{2+} and have a great capacity for binding Ca^{2+}, trap Ca^{2+} released through IP_3-induced Ca^{2+} release channels to prevent the Ca^{2+} concentration from reaching the threshold for activating Ca^{2+}-induced Ca^{2+} release channels. The proteins would serve as a functional barrier.

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