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Characteristics of Ca^{2+} Release Mechanisms from an Intracellular Ca^{2+} Store in Rabbit Coronary Artery

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To elucidate the Ca^{2+} release mechanisms in the rabbit coronary artery, arterial preparations were permeabilized with β-escin and changes in tension were measured under varying experimental conditions. Additionally, we investigated properties and distribution of two kinds of Ca^{2+} release mechanisms, Ca^{2+}-induced Ca^{2+} release (CICR) and IP_3-induced Ca^{2+} release (IICR). The results obtained were summarized as follows; ① When a rabbit coronary artery was incubated in a relaxing solution containing 30 μM β-escin for 40 min, sensitivity to externally added Ca^{2+} was much higher in β-escin permeabilized muscle than in intact preparations. The contractile effect of IP_3 in β-escin permeabilized muscle was also demonstrated ② Caffeine and IP_3 contracted coronary arteries were permeabilized with β-escin, but the amplitude of contraction was much larger in the presence of caffeine than of IP_3. ③ Intracellular heparin completely inhibited the contractions induced by IP_3, but not those by caffeine. On the other hand, procaine inhibited the responses to caffeine, but not those to IP_3. Ryanodine inhibited both the caffeine- and IP_3-induced contractions. ④ The amplitude of contractile responses was much larger to the maximal stimulation of CICR by applying caffeine than to the maximal stimulation of IICR by applying IP_3. After the maximal CICR stimulation by caffeine, the activation of IICR by IP_3 without the reloading of Ca^{2+} could no longer evoke contraction. On the other hand, after the maximal IICR activation, the activation of CICR could still evoke contraction although the amplitude of the contraction was smaller when compared with the case without the initial IICR stimulation. ⑤ Acetylcholine contracted coronary artery smooth muscles were permeabilized with β-escin. However, in the absence of added guanosine triphosphate (GTP), the responses were very small. Acetylcholine-induced contraction was inhibited by heparin, but not by procaine. From the above results, it may be concluded that there are two kinds of mechanisms of Ca^{2+} release, CICR and IICR, in the rabbit coronary artery smooth muscle cell. Also, whereas the CICR mechanism distributes on the membrane of the whole smooth muscle Ca^{2+} store, the IICR mechanism distributes only on a part of it.

Key Words: Ca^{2+} release mechanism, IICR, CICR, permeabilized rabbit coronary artery

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The increase in intracellular free Ca^{2+} concentration is a primary trigger of the physiological contraction of smooth muscle cells (Fay *et al.* 1979). Therefore, control of the cytoplasmic Ca^{2+} concentration is one of the major ways to regulate the strength of smooth muscle contraction, together with control of the sensitivity of the contractile protein to Ca^{2+}.

The Ca^{2+} that activates the contractile reaction in smooth muscle comes either from an extracellular medium or from an intracellular Ca^{2+} store (Bohr, 1973; Bolton, 1979). While the relative importance of these two Ca^{2+} sources in various types of contraction has not yet been precisely determined, it is clear that at least under certain cicumstances the Ca^{2+} released from the intracellular Ca^{2+} store plays a major role in evoking contraction of the smooth muscle cells (Somlyo *et al.* 1971).

It is important to know, therefore, about the detailed properties of the Ca^{2+} store and its Ca^{2+} release mechanisms. Intracellular Ca^{2+} store in smooth muscle cell is known as sarcoplasmic reticulum (SR) and Ca^{2+} release from SR is known to occur by a Ca^{2+}-induced Ca^{2+} release (CICR) mechanism (Itoh *et al.* 1981; Iino, 1987). It is also well known that caffeine releases Ca^{2+} from the SR by increasing the sensitivity of the CICR mechanism (Leitzen & van Breemen, 1984) and that ryanodine, a plant alkaloid that locks Ca^{2+} release channels on the SR in an open state (Fleischer *et al.* 1985) and depletes Ca^{2+} stored in the SR, inhibits the caffeine-induced Ca^{2+} release and contraction (Kanmura *et al.* 1988).

Endo *et al.* (1990) reported that two different kinds of Ca^{2+} release mechanisms, CICR and inositol 1, 4, 5-trisphosphate (IP_3)-induced Ca^{2+} release (IICR), are present in intracellular Ca^{2+} store of taenia caecum.

The present investigation was carried out to study the existence of two different kinds of Ca^{2+} release mechanisms and properties of the intracellular Ca^{2+} stores in the coronary artery smooth muscle cell in rabbits, using β-escin permeabilized preparations.

MATERIALS AND METHODS

Preparation of the coronary artery and tension recording

Small artery rings (100 μm wide and 2 mm long) of rabbit coronary artery smooth muscle were dissected and stretched to about 1.2 times the resting length. Isometric tension was measured with a force transducer in a chamber equipped with a solution exchanger.

Permeabilization with β-escin

After measuring steady contractions induced by high K^+, rings were incubated in a relaxing solution containing 4.5 mM MgATP, 0.2 mM EGTA, 1 μM carbonylcyanide 4-(trifluromethoxy) phenylhydrazone (FCCP) as a mitochondrial blocker, 1 μM leupeptin as a protease inhibitor (Kobayashi *et al.* 1989) and 2.7 μM calmodulin for 20~30 min. Cell permeabilization was achieved by incubation at 25℃ in 30 μM β-escin in the relaxing solution for 40 min.

Solutions

The normal external solution was a HEPES-buffered modified Krebs solution containing (mM): Na^+, 137.4; K^+, 5.9; Ca^{2+}, 1.2; Mg^{2+}, 1.2; Cl^-, 148.1; glucose, 11.5 and HEPES, 11.6 (pH 7.3 with NaOH at 20℃). The depolarizing, high K^+ (143 mM) solution was made by replacing Na^+ with an equivalent amount of K^+ and adding 43.8 mM sucrose in order to avoid cell swelling. The external Ca^{2+}-free solution always contained 2 mM EGTA. The normal relaxing solution was (mM): potassium methanesulfonate, 74.1; magnesium methanesulfonate, 2; MgATP, 4.5; EGTA, 2; creatine phosphate, 10; PIPES, 30 (pH 7.1 with KOH at 20℃). In activating or loading solutions, 10 mM EGTA was used, and a specified amount of calcium methanesulfonate was added to give a desired concentration of free Ca^{2+}. Ionic strength was kept constant at 0.2 M by adjusting the concentration of potassium methanesulfonate. The relaxing or loading solution

contained 2.7 μM calmodulin, a mitochondrial blocker (1 μM FCCP), and a protease inhibitor (1 μM leupeptin).

Materials

β-escin, heparin, calmodulin, FCCP, leupeptin, procaine, caffeine, inositol 1, 4, 5-trisphosphate (IP_3) and GTP were all purchased from Sigma. Potassium methanesulfonate, magnesium methanesulfonate and calcium methanesulfonate were purchased from Tokyo Pharmacia. All other reagents were of the highest grade commercially available.

RESULTS

Evidence of permeabilization by β-escin

The following findings are evidence of successful permeabilization of the cell membrane by β-escin.

After measuring steady contractions induced by high K^+ (Fig. $1A_1$), cell permeabilization was achieved by incubation at 25℃ in 30 μM β-escin in the relaxing solution for 40 min.

Sensitivity to externally added Ca^{2+} was much higher in β-escin-permeabilized muscle than in the intact preparations. pCa 6.5 produced 10% of maximum contraction, while in intact muscles pCa values had no effect on the force (Fig. $1A_2$ & $1B_1$).

The contractile effect of IP_3 (Fig. $1B_2$), mediated by the Ca^{2+} released from intracellular Ca^{2+} store, was also consistent with intracellular penetration of this polyvalent anion

Evidence of receptor coupling in β-escin permeabilized coronary artery

Fig. 2 illustrates the effects of acetylcholine on the contractile responses of a β-escin permeabilized coronary artery. When the SR was loaded with pCa 6.3 for 5min, acetylcholine contracted coronary artery smooth muscles permeabilized with β-escin (Fig. $2B_1$). However, in the presence of GTP, the responses were very large (Fig. $2B_2$). Acetylcholine-induced contraction was inhibited by heparin (Fig. $2B_3$), but not by procaine (Fig. $2B_4$).

The responses to IP_3 and caffeine

Fig. 3 illustrates the effects of IP_3 and caffeine on the contractility of β-escin permeabilized muscle. As shown in the Fig. 3, when the SR was loaded with Ca^{2+} (pCa 6.3 for 5~7 min), 20 mM caffeine and 40 μM IP_3

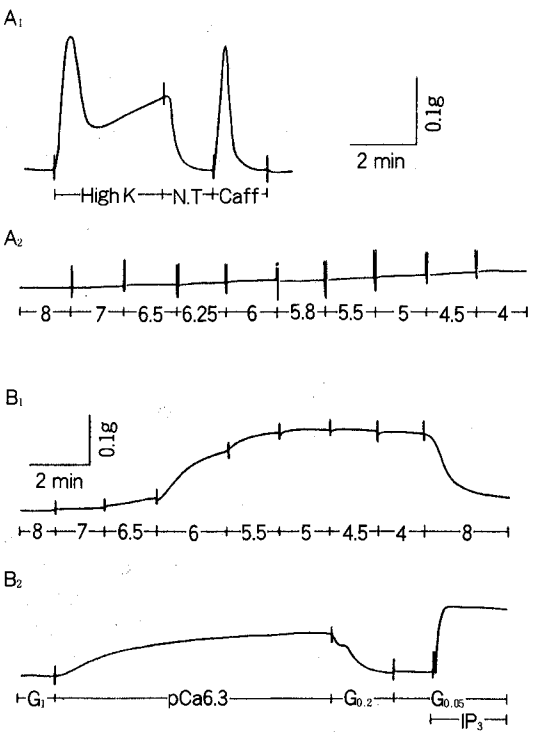

Fig. 1. *Contractile response of rabbit coronary artery permeabilized with a β-escin to IP_3. A_1 and A_2 are the responses of the muscle before permeabilization. A_1: high K^+ (154 mM) and caffeine (20 mM)-induced contractions. A_2: lack of contractile response of intact coronary artery to external Ca^{2+} (between pCa 8 and 4). B_1 and B_2 are the responses of the muscle following permeabilization with β-escin. B_1: the response of the permeabilized muscle of Ca^{2+}, showing the marked increase in sensitivity to external Ca^{2+} compared with the response to the same muscle prior to permeabilization (A_2). B_2: the response to IP_3 (40 μM). The SR was loaded with Ca^{2+} at pCa 6.3 for 5 min, followed by washout with 200 μM ($G_{0.2}$) and 50 μM ($G_{0.05}$) EGTA solutions. N-T: Normal Tyrode solution.*

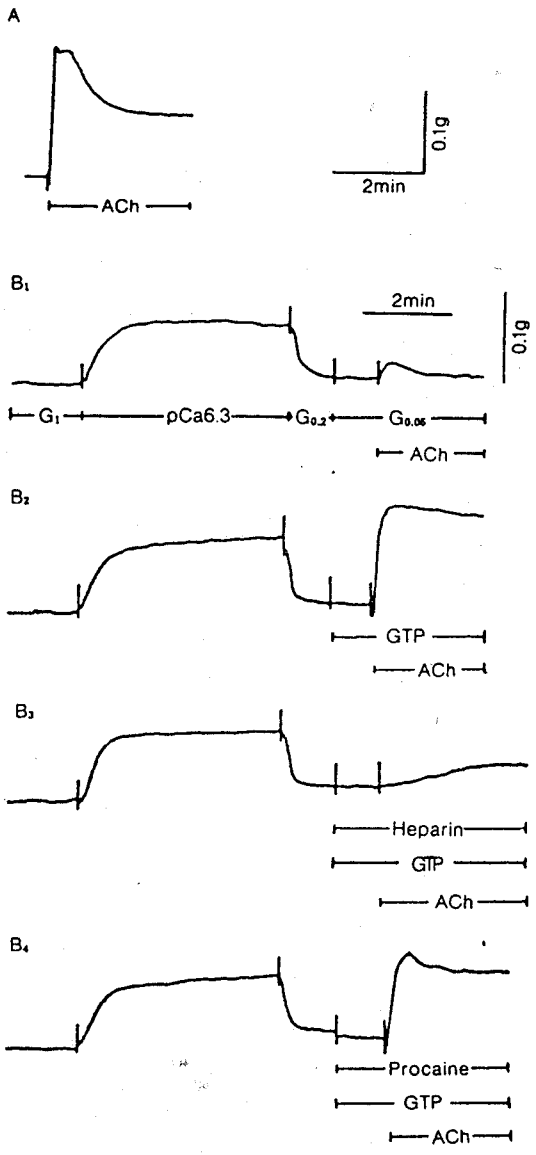

Fig. 2. *The effects of acetylcholine on the contractile responses of a β-escin-permeabilized and intact coronary artery. A: contractile response to 10^{-6}M acetylcholine (ACh) in intact preparation. B_1: contractile responses of β-escin permeabilized coronary artery to 10^{-6}M acetylcholine. B_2: contractile responses to 10^{-6}M acetylcholine in the presence of added 0.1 mM GTP. B_3: responses in the presence of 300 μg/ml heparin to acetylcholine. B_4: responses in the presence of 10 mM procaine to acetylcholine.*

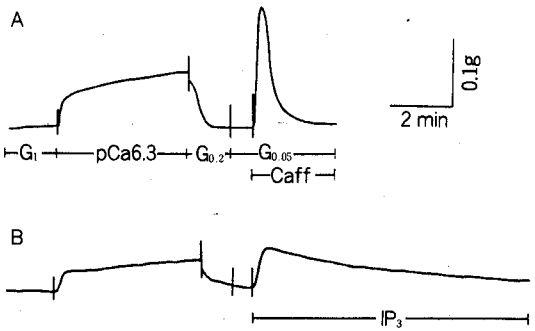

Fig. 3. *The effects of caffeine and IP_3 on the contractile response of a β-escin-permeabilized coronary artery. The SR was loaded with Ca^{2+} at pCa 6.3 for 5min, followed by washout with 200μM ($G_{0.2}$) and 50 μM ($G_{0.05}$) EGTA solutions, as shown in A. The strips were incubated in normal relaxing solution (G_1) for 10min between each protocol. A: the response to caffeine (20 mM). B: the response to IP_3 (40 μM).*

contracted coronary artery smooth muscles permeabilized with β-escin but the amplitude of the contraction was much larger in the presence of caffeine than of IP_3.

Characteristics of intracellular Ca^{2+} store in coronary artery

Fig. 4 illustrates the effects of heparin on the contractile responses of β-escin permeabilized muscle to caffeine and IP_3. Heparin (300 μg/ml), a specific blocker of IICR, inhibited an IP_3 (40 μM)-induced contraction. However, heparin did not block the caffeine (20 mM)-induced contraction.

Fig. 5 illustrates the effects of procaine on the contractile responses of a β-escin permeabilized muscle to caffeine and IP_3. Procaine (10 mM), a specific blocker of CICR, decreased caffeine (20 mM)-induced contraction, but did not inhibit the IP_3 (40 μM)-induced contraction.

Fig. 6 illustrates the effects of pretreatment with ryanodine on the contractile responses of a β-escin permeabilized muscle to caffeine and IP_3. Pretreatment with ryanodine (1 μM), a plant alkaloid that fixes CICR channels on the SR in an open state, inhibited both caf-

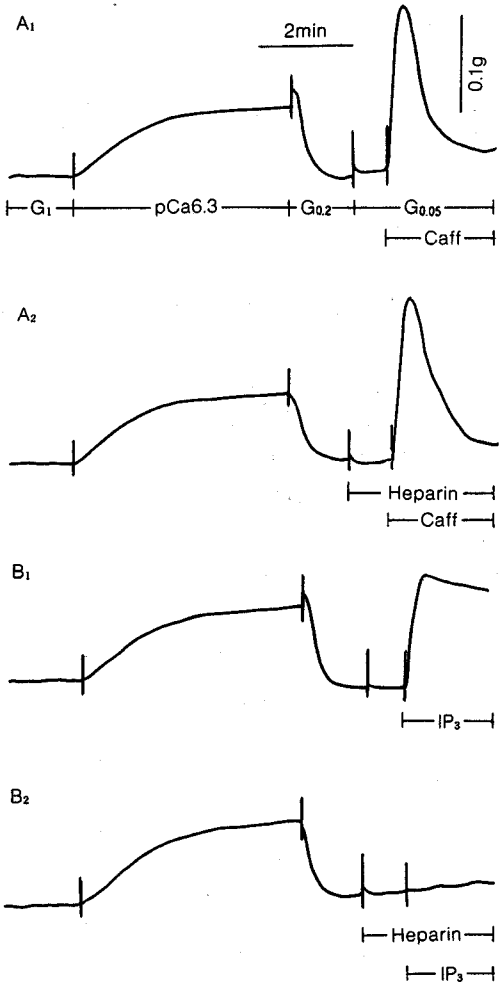

Fig. 4. *The effects of heparin on the contractile responses of a β-escin-permeabilized coronary artery to caffeine (Caff) and IP_3. The SR was loaded with Ca^{2+} at pCa 6.3 for 5min, followed by washout with 200 μM ($G_{0.2}$) and 50 μM ($G_{0.05}$) EGTA solutions, as shown in A_1. The strips were incubated in a normal relaxing solution (G_1) for 10min between each protocol. A_1 and A_2: effects of 20 mM caffeine on contractile responses with and without heparin (300 μg/ml), respectively. B_1 and B_2: effects of 40 μM IP_3 on contractile responses with and without heparin (300 g/ml), respectively.*

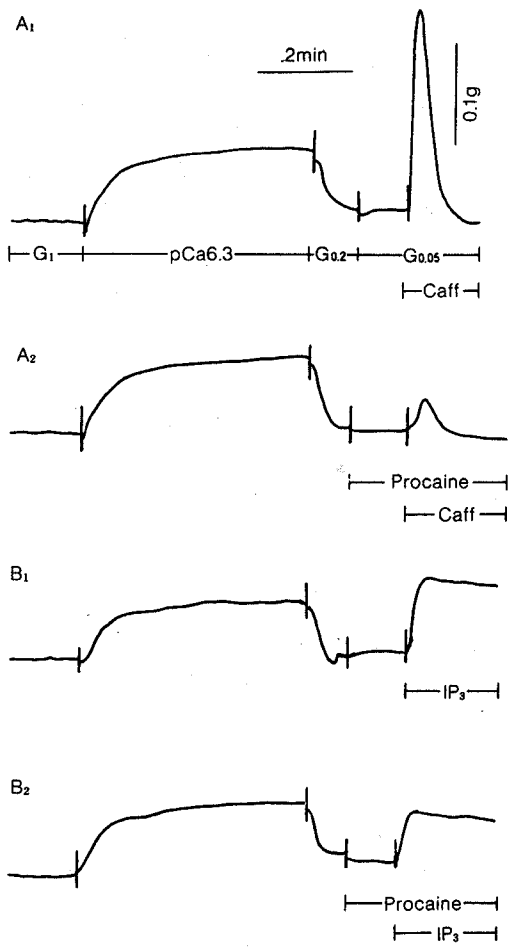

Fig. 5. *The effects of procaine on the contractile responses of a β-escin-permeabilized coronary artery to caffeine (Caff) and IP_3. The SR was loaded with Ca^{2+} at pCa 6.3 for 5min, followed by washout with 200 μM ($G_{0.2}$) and 50 μM ($G_{0.05}$) EGTA solutions, as shown in A_1. The strips were incubated in a normal relaxing solution (G_1) for 10min between each protocol. A_1 and A_2: effects of 20 mM caffeine on contractile responses with and without procaine (10 mM), respectively. B_1 and B_2: effects of 40 μM IP_3 on contractile responses with and without procaine (10 mM), respectively.*

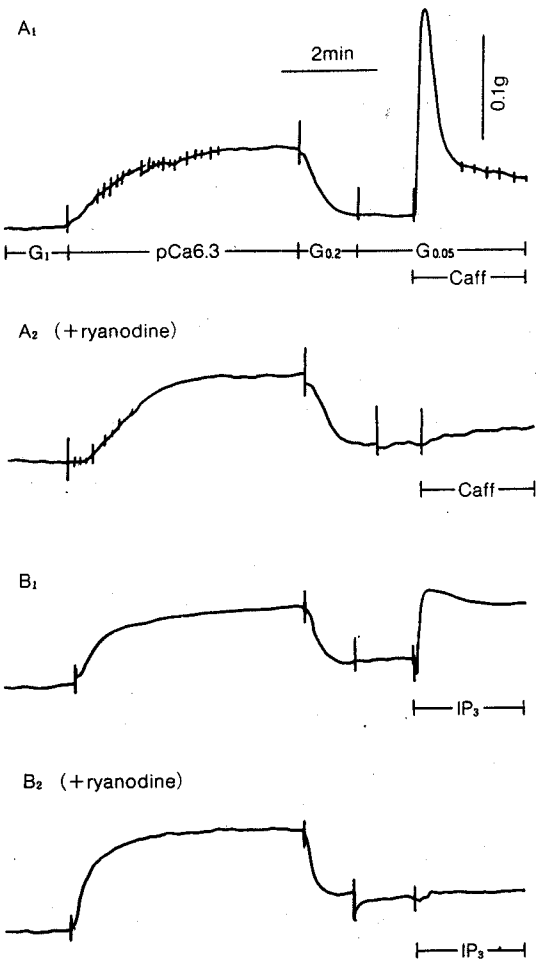

Fig. 6. *The effects of ryanodine on the contractile responses of a β-escin-permeabilized coronary artery to caffeine (Caff) and IP_3. The SR was loaded with Ca^{2+} at pCa 6.3 for 5min, followed by washout with 200 μM ($G_{0.2}$) and 50 μM ($G_{0.05}$) EGTA solutions, as shown in A_1. The strips were incubated in a normal relaxing solution (G_1) for 10 min between each protocol. A_1 and A_2: effects of 20 mM caffeine on contractile responses with and without pretreatment of ryanodine (1 μM), respectively. B_1 and B_2: effects of 40 μM IP_3 on contractile responses with and without pretreatment of ryanodine (1 μM), respectively.*

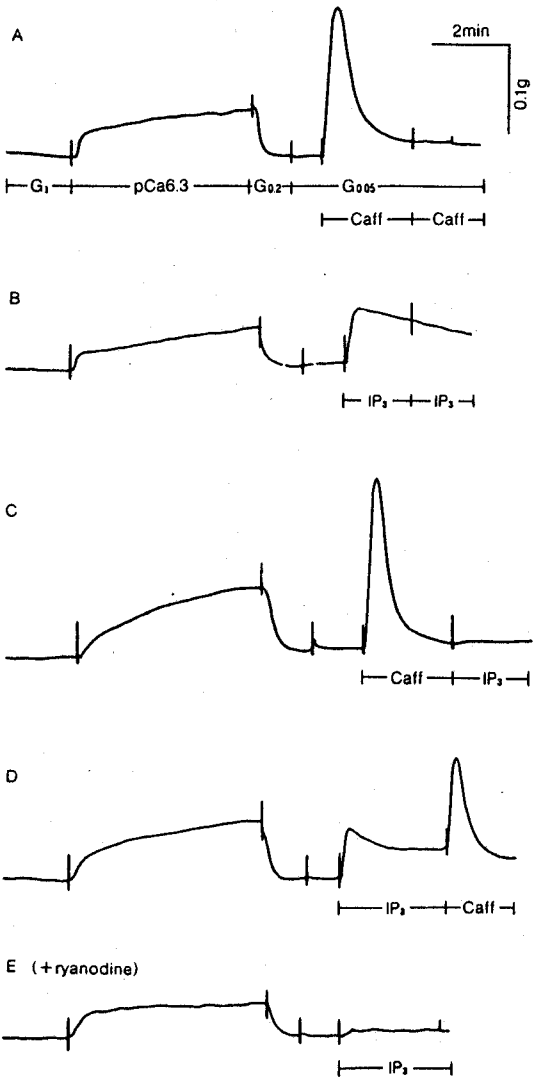

Fig. 7. *Distribution of the Ca^{2+} release mechanisms in the coronary artery. The SR was loaded with Ca^{2+} at pCa 6.3 for 5min, followed by washout with 200 μM ($G_{0.2}$) and 50 μM ($G_{0.05}$) EGTA solutions, as shown in A_1. The strips were incubated in a normal relaxing solution (G_1) for 10min between each protocol. A and B: the response to double treatment of 20 mM caffeine or 40 μM IP_3, respectively. C: the response (after caffeine pretreatmeant) to IP_3. D: the response (after IP_3 pretreatment) to caffeine. E: the response after the pretreatment of 1 μM ryanodine to IP_3.*

feine (20 mM)- and IP_3 (40 μM)-induced contractions.

Distribution of the Ca^{2+} release mechanisms

Fig. 7 shows distribution of the Ca^{2+} release mechanisms in the coronary artery smooth muscle cell.

As shown in Fig. 7, application of 20 mM caffeine or 40 μM IP_3 to the bath solution caused a transient increase of tension in the muscle cell which returned to the resting level in the presence of caffeine or IP_3. The second application of caffeine or IP_3 in the same tissue did not cause any change in tension (Fig. 7A, 7B). The amplitude of contraction was much larger in the applying caffeine than in the applying IP_3. After the maximal CICR stimulation, the activation of IICR by IP_3 without reloading of Ca^{2+} elicited no tension elevation (Fig. 7C). On the other hand, after the maximal IICR stimulation, the activation of CICR could still evoke a tension elevation although the amplitude of tension was smaller when compared with the case without initial IICR stimulation (Fig. 7D). Pretreatment with ryanodine (1 μM) inhibited an IP_3 (40 μM)-induced contraction (Fig. 7E).

DISCUSSION

Permeabilization of the smooth muscle with crude saponin has been a valuable technique for the direct control of cytosolic solute composition (Endo *et al.* 1977). The smooth muscles permeabilized by this method also retain the G protein/phospholipase C/IP_3 cascade (Kobayashi *et al.* 1988a, 1988b) and the sensitivity of the IP_3 receptor to heparin (Kobayashi *et al.* 1988a). However, saponin permeabilization uncouples all of the receptor (Itoh et al. 1983; Somlyo *et al.* 1985). Smooth muscles permeabilized with Staphylococcal α-toxin retain their responsiveness to muscarinic (Kitazawa *et al.* 1989) and to α_1-adrenergic agonists (Nishimura *et al.* 1988; Kitazawa *et al.* 1989), but this method allows only lower molecular weight compounds than heparin to penetrate the membrane (Ahnert-Hilger and Gratzl, 1988). Therefore, we have used a commercially, readily available, saponin ester, β-escin, that makes the cell membrane permeable to higher molecular weight solutes such as heparin and calmodulin, and retains coupled receptors (Kobayashi *et al.* 1989). The following results provide evidence of the successful permeabilization of the cell membrane by -escin. First, sensitivity to externally added Ca^{2+} was much higher in -escin-skinned muscle than in the intact preparation (Fig. 1). Secondly, the contractile effect of IP_3 was also consistent with intracellular penetration of this polyvalent anion (Fig. 1 & 3). And muscarinic agonist, acetylcholine, contracted coronary artery smooth muscles permeabilized with β-escin (Fig. 2). However, in the absence of added GTP, the responses were very small. The contraction induced by activation of muscarinic receptors was inhibited by the heparin, specific blocker of IP_3-induced Ca^{2+} release (Iino, 1987). This presumably reflects the GTP requirement for the coupling, via G-protein, of the receptor to the effector (phospholipase C; Kitazawa *et al.* 1989) and suggests that smooth muscle permeabilized by β-escin retain the G-protein/phospholipase C/IP_3 cascade and the sensitivity of the IP_3 receptor to heparin.

Endo *et al.* (1990) reported that two different kinds of Ca^{2+} release mechanisms, CICR and IICR, have been present in intracellular Ca^{2+} store of taenia caecum. And it is well known that caffeine and IP_3 release Ca^{2+} from the Ca^{2+} store (Leitzen and van Breemen, 1984). In the present study, we have tested the effect of caffeine and IP_3 on the release of Ca^{2+} from intracellular Ca^{2+} store in order to confirm whether two kinds of mechanisms of Ca^{2+} release were existant or not. When the SR was loaded with Ca^{2+} (pCa 6.3 for 5~7min), caffeine and IP_3 contracted coronary artery smooth muscles permeabilized with β-escin (Fig. 3). Intracellular heparin completely inhibited the contractions induced by IP_3, but not those by caffeine (Fig. 4). On the other hand, procaine inhibited the responses to caffeine, but not those to IP_3 (Fig. 5). Ryanodine inhibited the caffeine- and IP_3-induced contraction (Fig. 6). It is well known that caffeine releases Ca^{2+} from the SR by increasing the sensitivity

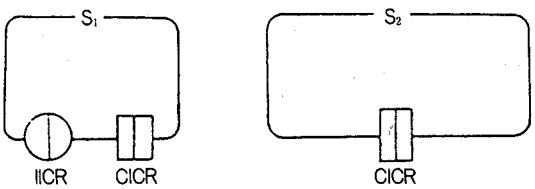

Fig. 8. *A scheme of compartments of coronary artery Ca^{2+} store. S_1: intracellular Ca^{2+} store with both Ca^{2+}-induced Ca^{2+} release (CICR) and IP_3-induced Ca^{2+} release (IICR) mechanisms. S_2: intracellular Ca^{2+} store only with Ca^{2+}-induced Ca^{2+} release (CICR) mechanism.*

of the CICR mechanism (Lei zen and van Breemen, 1984) and that CICR is inhibited by procaine (Endo, 1985). Ryanodine is a plant alkaloid that lockes Ca^{2+} release channels on the SR in an open state (Fleisher *et al.* 1985) and depletes Ca^{2+} stored in the SR (Kanmura *et al.* 1988). Therefore, our results indicate that two different kinds of Ca^{2+} release mechanisms, CICR and IICR, were shown to be present in the coronary artery smooth muscle of a rabbit.

Fig. 7 shows contractile responses to maximal CICR and IICR stimulations of intracellular Ca^{2+} store in a β-escin permeabilized coronary artery. In order to know whether concentration of caffeine or IP_3 used is enough to release Ca^{2+} through the Ca^{2+} release mechanism, we measured the effects of caffeine and IP_3 on the muscle contraction. Application of 20 mM caffeine or 40 μM IP_3 to the bath solution caused a transient increase of tension, and it returned to the resting level in the presence of caffeine or IP_3. The second application of caffeine or IP_3 in the same tissue did not cause any change in tension, which suggest the complete release of Ca^{2+} through the Ca^{2+} release mechanism by treatment of 20 mM caffeine or 40 μM IP_3 (Fig. 7A, 7B). It is clearly seen that the maximal stimulation of CICR by applying 20 mM of caffeine caused a much larger amplitude of contraction than the maximal stimulation of IICR by applying 40 μM IP_3. After the maximal CICR stimulation, the activation of IICR by IP_3 without reloading of Ca^{2+} elicited no tension elevation (Fig. 7C). On the other hand, after the maximal IICR stimulation, the activation of CICR could still evoke a tension elevation although the amplitude of tension was smaller when compared with the case without initial IICR stimulation (Fig. 7D). Pretreatment with ryanodine inhibited IP_3-induced contraction (Fig. 7E). These results are consistent with the idea that the CICR mechanism distributes on the membrane of the whole smooth muscle Ca^{2+} store, but the IICR mechanism distributes only on a part of it (Fig. 8). Endo *et al.* (1990) reported that IICR mechanism distributes on the membrane of the whole smooth muscle Ca^{2+} store in guinea-pig taenia caeci, but only on part of the CICR mechanism. Discrepancy between our results and the report of Endo *et al.* (1990) may be due to heterogenous distribution of the CICR and the IICR mechanisms between the different types of smooth muscle (Iino *et al.* 1988).

In conclusion, there are two kinds of mechanisms of Ca^{2+} release, CICR and IICR, in rabbit coronary artery smooth muscle cell and the CICR mechanism distributes on the membrane of the whole smooth muscle Ca^{2+} store, unlike the IICR mechanism which only distributes on part of it.

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