Calcium-induced Calcium Release
Mechanism in Guinea Pig Taenia Caeci

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ABSTRACT Fura-2 was used to measure the amount of Ca released from the intracellular Ca store of a saponin-skinned smooth muscle fiber bundle of the guinea pig taenia caeci (width, 150–250 μm) placed in a capillary cuvette at 20–22°C. The amount of Ca actively loaded into the store was assayed when released by the application of 50 mM caffeine and/or 10 μM inositol 1,4,5-trisphosphate (IP₃) in the absence of ATP, and was found to have a biphasic dependence on the loading [Ca²⁺] with a peak near pCa 6. After Ca loading at pCa 6, IP₃ released almost all the releasable Ca, whereas caffeine discharged Ca from only ~40% of the store. The maximum amount of Ca in the store was some 220 μmol/liter cell water. Ca in the caffeine-releasable store was released approximately exponentially to zero with time when Ca²⁺ was applied in the absence of ATP, and the rate constant of the Ca-induced Ca release (CICR) increased steeply with the concentration of Ca²⁺ applied. Increase in [Mg²⁺] (0.5–5.0 mM) or decrease in pH (7.3–6.7) shifted the relation between pCa and the rate of CICR roughly in parallel toward the lower pCa. An adenine nucleotide increased the rate of the CICR, but it did not change the range of effective [Ca²⁺]. 5 mM caffeine greatly enhanced the CICR mechanism, making it ~30 times more sensitive to [Ca²⁺]. However the drug had no Ca-releasing action in the absence of Ca²⁺. Procaine in millimolar concentrations inhibited the rate of the CICR. These properties are similar to those of the skeletal muscle CICR and ryanodine receptor channels. Rates of the CICR under a physiological ionic milieu were estimated from the results, and a [Ca²⁺] >1 μM was expected to be necessary for the activation of the Ca release. This Ca sensitivity seems too low for the CICR mechanism to play a primary physiological role in Ca mobilization, unless assisted by other mechanisms.

INTRODUCTION
Caffeine causes transient contractures of smooth muscle bundles in the absence of extracellular calcium ions (Endo et al., 1977; Bond et al., 1984). In skinned smooth muscle fiber bundles, caffeine initiates transient tension development due to Ca²⁺ mobilized within the cells (Endo et al., 1977; Itoh et al., 1981; Saida, 1982). Release of Ca²⁺ from skinned cells by the drug has been directly measured either by radioactive Ca isotope (Stout and Diecke, 1983; Yamaoto and van Breemen, 1985) or by a
fluorescent Ca indicator (Iino, 1987). These results clearly indicate that smooth muscle cells have a caffeine-releasable intracellular Ca store.

In striated muscles, caffeine releases Ca from the sarcoplasmic reticulum (SR) through activation of the Ca-induced Ca release (CICR) mechanism (Endo, 1977, 1985). By analogy, similar Ca-induced Ca release mechanism has been thought to be present in smooth muscle cells. And this view has been supported by some indirect evidence obtained in skinned fiber experiments, such as a decline of Ca uptake capacity of the store above an optimal Ca\(^{2+}\) concentration (Itoh et al., 1981; Saida, 1982). However, direct evidence for the presence of the Ca-induced Ca release in smooth muscle is yet to be obtained. If such Ca release mechanism is present, then it is important to determine its properties and physiological role, because agonists seem to release Ca from the same store as caffeine does (Endo et al., 1980; Casteels and Raeymaekers, 1979; Bond et al., 1984; Iino et al., 1988), and the mechanism of physiological Ca mobilization in smooth muscle has not been fully understood.

The present study characterizes Ca uptake and Ca release mechanisms of the intracellular Ca store in saponin-skinned fiber bundles of the guinea pig taenia caeci using a method based on the fluorescent Ca indicator, fura-2 (Grynkiewicz et al., 1985), to measure Ca release. The experimental protocol to study Ca release mechanism was designed in such a way that the release of Ca from the store took place in the absence of ATP and in the presence of a high concentration of a Ca buffer. Therefore, the properties of Ca release could be studied without being affected either by any change in the rate of Ca uptake or by a secondary Ca release due to transient increase in the Ca\(^{2+}\) concentration in the vicinity of the Ca store. The present study demonstrates that caffeine-releasable store, which constitutes about 40% of the total Ca store in guinea pig taenia caeci, has a Ca-induced Ca release mechanism, the properties of which are similar to those of the skeletal muscle counterpart. It also shows that unless enhanced by caffeine, Ca\(^{2+}\) concentrations above some 1 \(\mu\)M are required for the activation of this Ca release mechanism.

**METHODS**

**Outline of the Method**

Skinned fiber bundles of the guinea pig taenia caeci were placed in a glass capillary cuvette through which solutions can be rapidly flushed. Fiber bundles were treated with Ca\(^{2+}\) buffered with EGTA (ethyleneglycol-bis[\(\beta\)-aminoethyl ether] \(N,N',N'\)-tetraacetic acid) under various conditions so that the Ca store would take up Ca\(^{2+}\). Ca\(^{2+}\), ATP, and EGTA were then withdrawn, and a high concentration of caffeine and/or IP\(_3\) was applied to release Ca\(^{2+}\) from the store in the presence of fura-2, a fluorescent Ca indicator (Grynkiewicz et al., 1985). Almost all the Ca released would bind to fura-2 because of the ligand's high affinity to Ca, and the resulting change in the fluorescence intensity of the dye was monitored by a microfluorometry to measure the amount of released Ca.

**Fluorescence Measurement and Data Collection**

The apparatus was built on an epifluorescence microscope (BHS-RFK, Olympus, Tokyo, Japan). Light from a 75-W xenon lamp powered by a regulated direct current supply (L2174 and C2177, Hamamatsu Photonics, Hamamatsu, Japan) was passed through either a 340-nm interference filter (half bandwidth, \(\lambda_{1/2} \sim 9\) nm) or, in some experiments, a 380-nm filter.
calcium-induced calcium release in smooth muscle (λ<sub>r</sub> = 24 nm), reflected by a dichroic mirror and focused onto the capillary cuvette by an objective (DPlan Apo 20× UV, NA = 0.70, Olympus). Illumination spot size was 0.8 mm in diameter. The dye fluorescence was collected by the same objective, passed through both the dichroic mirror and an interference filter (494 nm, λ<sub>i</sub> = 14 nm) or a wide-band pass interference filter (500 nm, λ<sub>i</sub> = 40 nm) and was directed to a photomultiplier tube (R647, Hamamatsu Photonics). Interference filters were purchased from Vacuum Optics Corporation of Japan (Tokyo). In the later course of the study, a custom-made light source and photometer system (CAX-100, Nihon Bunko Kogyo, Tokyo, Japan) was used. Important differences were the brighter Xe lamp (150 W) and the use of a monochromator instead of the interference filter for the selection of the excitation wavelength (340 nm).

In the current experiments, fura-2 at a fixed concentration was freshly applied during each Ca assay. Because Ca assay was carried out in the absence of ATP, there was no movement of the preparation upon release of Ca from the store. Therefore, the current method is free from the problems usually encountered in the free Ca concentration measurement in fura-2-loaded intact cells, such as loss or bleaching of the dye and movement artifacts. For this reason, the fluorescence intensity of fura-2 was usually measured with single-wavelength excitation at 340 nm. Because CAX-100 has a facility to carry out double-wavelength excitation, the ratio of the fluorescence intensity with 340 nm excitation to that with 360 nm excitation was measured in some experiments, and the results were similar to those of single-wavelength excitation.

The photocurrent signal of the photomultiplier tube was fed into a transient recorder (KE-8200, Kawasaki Electronica, Tokyo, Japan) and 4 k words of 12-bit data were sampled every 20 μs. The initiation of data sampling and the opening of the shutter (50 ms/measurement) were synchronized in such a way that the first half of the sampling was carried out when the shutter was closed, the latter half while the shutter was open. The digital data were immediately transferred to a microcomputer (PC98XA, Nihon Denki, Tokyo, Japan). The fluorescence intensity was averaged, plotted on a graphic display terminal, and stored on a magnetic disk for the later analysis. The data collection routine was repeated with 5- or 6-s intervals.

The transient recorder, the shutter, and the pumps were all in the control of the computer via a digital input/output interface board and electronic stimulators.

**Calibration**

Fig. 1 shows the calibration of the fluorescence measuring system. In Fig. 1 A, fura-2-containing solutions with various concentrations of Ca, added as indicated in the figure, were introduced into the capillary cuvette at the arrows and the total fluorescence intensity was measured. In Fig. 1 B, the extra light signal (FI) above the fluorescence intensity in the presence of 1 mM EGTA and no added Ca was normalized by the maximum FI and plotted against the added Ca concentration (C<sub>a</sub>) offset by C<sub>cno</sub> (see below). The dotted line shows the theoretical curve of the following equation:

\[
\text{FI} = \left[ C_a + C_f + K_d - \left( (C_a + C_f + K_d)^3 \right) \right] / (2C_d),
\]

where \( C_a = C_c + C_cno \).

This equation was derived with the following assumptions: (a) Change in the fluorescence intensity is proportional to the concentration of Ca·fura-2 complex. (b) Ca and fura-2 bind with 1:1 stoichiometry with a dissociation constant of \( K_d \). (c) The total concentration of fura-2 is \( C_f \). (d) The level of Ca contamination in the solutions is \( C_cno \). From these assumptions,

\[
\text{FI} = [\text{Ca} \cdot \text{fura-2}] / [\text{fura-2}]_{\text{total}},
\]

\[
[\text{Ca} \cdot \text{fura-2}] = [\text{Ca}^2+] [\text{fura-2}] / K_d.
\]
$$\text{[Ca-fura-2]} + \text{[fura-2]} - \text{[fura-2]}_{\text{free}} = C_t, \quad (4)$$

$$\text{[Ca-fura-2]} + \text{[Ca}^{2+}] = \text{[Ca]}_{\text{total}} = C_{c} + C_{\text{free}} = C_{x}. \quad (5)$$

Eq. 1 is obtained if Eqs. 3–5 are solved for \([\text{Ca-fura-2}]\), and the expression is inserted into Eq. 2.

A least-square fit of Eq. 1 of the data points was obtained with \(K_d = 270 \text{ nM}\), \(C_t = 40.4 \mu\text{M}\), and \(C_{\text{free}} = 4.8 \mu\text{M}\). The value for \(K_d\) is similar to that reported by Grynkiewicz et al. (1985).

In the skinned fiber experiments the largest change in the fluorescence intensity was usually one fifth to one third of the maximum response, i.e., within the virtually linear range of the calibration.

The calibrations also provided an estimate of fura-2 concentration \((C_p)\). Together with the optical density of fura-2 in the absence of both Ca\(^{2+}\) and Mg\(^{2+}\) (solution G1RMg0, Table I)

![Figure 1](image-url)  
**Figure 1.** Calibration of the fluorescence intensity of fura-2. (A) Fluorescence intensity of fura-2 in the capillary cuvette with various concentrations of Ca. Excitation at 340 nm. At first arrow, fura-2 without added Ca was flushed into the cuvette. This was followed by flushes of solutions containing 10, 20, 30, 40, 50, and 100 \(\mu\text{M}\) total Ca, then fura-2 solution containing 1 mM EGTA was introduced. At the end fura-2 was washed away (open arrow). (B) Extra fluorescence signal \((\text{FI})\) above the intensity of fluorescence with 1 mM EGTA was plotted against added Ca concentration. FI was normalized by the maximum FI obtained at 100 \(\mu\text{M}\) Ca. For explanation of dotted curve, see text.

measured by a spectrophotometer (type 340, Nissei Sangyo, Tokyo, Japan), the molar extinction coefficient of the dye at 362 nm was estimated to be 31.5 mM\(^{-1}\)cm\(^{-1}\) (mean of three determinations, 30.8, 31.7, and 32.1 mM\(^{-1}\)cm\(^{-1}\)), which is within the range (30–40 mM\(^{-1}\)cm\(^{-1}\)) reported by Grynkiewicz et al. (1985). Concentrations of stock solutions of fura-2 were determined based on this molar extinction coefficient.

**Preparation of Skinned Fiber Bundle and Capillary Cuvette**

Male guinea pigs weighing 200–400 g were stunned and bled. Taenia, with a small amount of underlying circular muscle, was dissected from the caecum and stored in a normal external solution (in millimolar, NaCl, 150; KCl, 4; CaMg\(_2\) (calcium methanesulfonate), 2; MgMg\(_2\), 1; Hepes (N-2-hydroxyethylpiperazine-N'-ethanesulfonic acid), 5; pH 7.4 adjusted with
tris(hydroxymethyl) aminomethane. Thin fiber bundles devoid of both circular muscle and peritoneum, 150–250 µm in width, were carefully dissected either in a relaxing solution (G1, see Table I) or in a Ca-free external solution in which CaM²⁺ in the normal external solution was substituted with equimolar EGTA. Fiber bundles were tied with silk filaments at the both ends to tungsten wires (diameter, 100 µm; length, 15 mm) coated with Teflon wax. The length of the preparation between the knots was ~5 mm.

The fiber bundle attached to the metal wire was inserted into a glass capillary (internal diameter, 0.4 mm; length, 32 mm). Due to its gentle curvature, the wire secured its position in the capillary, which was in turn mounted in a groove cut on a Perspex holder with a glass bottom. The space around the capillary was filled with a low-fluorescent immersion oil, and a coverslip was laid on the top. Circulating water beneath the glass bottom kept the temperature around the capillary cuvette between 20 and 22°C. The temperature was monitored by a fine thermocouple placed within 1 mm of the capillary. The capillary cuvette holder was then mounted on the stage of the microscope.

One end of the capillary cuvette was connected to a thin stainless tubing via a silicone tubing. The other end was linked to two peristaltic pumps arranged in parallel. One of the pumps (MHRE/mark-4, Watson-Marlow Ltd., Falmouth, UK) was used to suck 180 µl of solutions (three times the dead space) in 1 s to rapidly change the solution in the capillary.

### Table I

**Composition of the Solutions**

| Name      | EGTA | CaEGTA | MgMg²⁺ | KMg | ATP | AMP | POPC | Mg²⁺ |
|-----------|------|--------|--------|-----|-----|-----|------|------|
| G1        | 1    | 0      | 5.54   | 108.6 | 4.76 | 0   | 1.5  |
| G10       | 10   | 0      | 5.90   | 81.8  | 4.74 | 0   | 1.5  |
| CaG10     | 0    | 10     | 5.50   | 82.0  | 4.80 | 0   | 1.5  |
| G0RMg0    | 0    | 0      | 142.1  | 0     | 0   | 0   | 0    |
| ASSAY*    | 0    | 0      | 84.1   | 0     | 0   | 0   | 0    |
| DEPLETIE* | 1    | 0      | 81.0   | 0     | 0   | 0   | 0    |
| G1R       | 1    | 0      | 1.54   | 154.5 | 0   | 0   | 1.5  |
| G10R      | 10   | 0      | 1.90   | 107.1 | 0   | 0   | 1.5  |
| CaG10R    | 0    | 10     | 1.50   | 107.5 | 0   | 0   | 1.5  |
| G10RMg0   | 10   | 0      | 111.8  | 0     | 0   | 0   | 0    |
| CaG10RMg0 | 0    | 10     | 112.0  | 0     | 0   | 0   | 0    |
| G10RMg0LHpH | 10  | 0      | 111.8  | 0     | 0   | 0   | 0    |
| CaG10RMg0LHpH | 0  | 10     | 118.3  | 0     | 0   | 0   | 0    |
| G10RMg0HpH | 10  | 0      | 104.6  | 0     | 0   | 0   | 0    |
| CaG10RMg0HpH | 0  | 10     | 105.2  | 0     | 0   | 0   | 0    |
| G10R-A2   | 10   | 0      | 3.62   | 97.5  | 0   | 2   | 1.5  |
| CaG10R-A2 | 0    | 10     | 3.20   | 97.9  | 0   | 2   | 1.5  |
| G10RMg0.5-A2 | 10 | 0      | 1.99   | 100.0 | 0   | 2   | 0.5  |
| CaG10RMg0.5-A2 | 0  | 10     | 1.81   | 100.5 | 0   | 2   | 0.5  |
| G10RMg5-A2 | 10  | 0      | 8.15   | 87.0  | 0   | 2   | 5.0  |
| CaG10RMg5-A2 | 0  | 10     | 6.91   | 87.7  | 0   | 2   | 5.0  |

Concentrations are in millimolar. All solutions contained 20 mM PIPES (piperazine-N,N'-bis(2-ethanesulfonic acid)) and 20 mM NaN₄. *ASSAY and DEPLETE contained both 50 mM caffeine and 25 mM AMP. pH was adjusted to 7.0 (except for 6.7 and 7.3) at 20°C with KOH. Mg²⁺ concentration was estimated by the numerical solution of multiequilibrium between metals and ligands in the solution, and 0 means nominally Mg free. When ATP was present, MgATP²⁺ concentration was calculated to be 4.0 mM.
The other pump (Minipuls II, Gilson France S.A., Villiers le Bel, France) was used to induce a slow continuous flow (1 μl/s) through the cuvette.

The fiber bundle was treated with 50 μg saponin/ml in a relaxing solution (G1) for 30 min to make perforations in the surface membrane (Ohtsuki et al., 1978; Endo and Iino, 1980; Endo et al., 1977; Iino, 1981).

**Solutions and Experimental Protocol**

The composition of the solutions used is listed in Table 1. Experimental solutions of various pCa were prepared by mixing two solutions containing 10 mM EGTA without Ca and both 10 mM EGTA and 10 mM Ca (Table II, fura-2 was absent from these solutions). Ionic constituents were computed by solving multiequilibrium equations using binding constants compiled by Martell and Smith (1974-1982). The apparent binding constant thus obtained for Ca-EGTA was $10^{4.39} \text{M}^{-1}$ at 20°C, pH 7.0, and 1.5 mM Mg$^{2+}$. In place of binding constants for H-, Mg-, and Ca-AMPOPCP ($\beta,\gamma$-methyleneadenosine 5'-triophosphate), those of $\beta,\gamma$-imidoadenosine 5'-triphosphate (Pettit and Siddiqui, 1976) were used. Stability constants for K- and Na-AMPOPCP were assumed to be the same as those of ATP.

| Condition          | 7.5 | 7.0 | 6.5 | 6.0 | 5.7 | 5.5 | 5.0 | 4.5 | 4.0 |
|--------------------|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Loading            |     |     |     |     |     |     |     |     |     |
| RMg0              | 0.71| 1.96| 4.34| 7.09| 8.29| 8.85| 9.61| 9.90|     |
| RMg0LpH           |     | 0.58| 1.63| 3.81|     | 6.60| 8.61| 9.54| 9.94|
| RMg0H2pH          |     | 4.89| 7.52| 9.06|     | 9.68| 9.91|     |     |
| R & R-A2          | 0.69| 1.89| 4.24| 7.00|     | 8.81| 9.60| 9.91|     |
| RMg0.5-A2         |     | 1.93| 4.31| 7.06|     | 8.84| 9.62| 9.94|     |
| RMg5-A2           |     | 1.76| 4.03| 6.81|     | 8.71| 9.56| 9.89|     |

Concentrations are in millimolar. Ca-containing solutions of various pCa were prepared by mixing G10 and CaG10 solutions in such a ratio that the total Ca concentration was equal to the values shown in this table.

Na$_2$ATP was obtained from Boehringer Mannheim (FRG), saponin from ICN Pharmaceuticals Inc. (Cleveland, OH), fura-2 from Molecular Probes, Inc. (Eugene, OR), EGTA from Dojindo Laboratories (Kumamoto, Japan). AMPOPCP and inositol 1,4,5-trisphosphate were purchased from Sigma Chemical Co. (St. Louis, MO). All the other chemicals were of the highest reagent grade.

The following explains the sequence of solution changes to study the properties of Ca uptake by the store in skinned fiber bundles. The names of the solutions (cf. Table I), duration of application, and their purposes are described. The suffix -F denotes that the solution contains fura-2 (25–45 μM).

(a) G1, 60–120 s. Preincubation in a relaxing solution with a low concentration of EGTA in the presence of MgATP. (b) Ca loading solution, various periods of time. (c) G10R, 60 s. Removal of Ca$^{2+}$ and ATP. (d) G1R, 60 s. Reduction of the EGTA concentration. (e) G1R-F, 60 s. Introduction of fura-2. (f) G0RMg0-F, 60 s. Withdrawal of both EGTA and Mg in the presence of fura-2. (g) ASSAY-F, 70 s. Application of 50 mM caffeine and/or 10 μM IP$_3$ together with 25 mM AMP to rapidly release Ca from the store. (h) DEPLETE, 60 s. Reintroduction of 1 mM EGTA in the continued presence of caffeine and/or IP$_3$ and AMP to thoroughly wash out Ca released by caffeine. Fura-2 was withdrawn. (i) G1R, 60 s. To wash out caffeine and/or IP$_3$ and AMP. Experiments can be repeated by going back to procedure a.
To study properties of the Ca release mechanism, we carried out Ca loading under a constant condition, and a Ca-releasing procedure was inserted between the Ca loading and the Ca assay procedure. The Ca-releasing test procedure was carried out in the absence of ATP to avoid Ca uptake. The amount of Ca released during the test procedure was estimated by the decrease in the Ca response for the assay in comparison with that of the control, i.e., run without step d2 (see below).

In practice the experimental protocol described above was modified as follows. Only the altered steps are described. Ca loading of protocol b was always carried out at pCa 6 for 3 min. Total EGTA concentration was 1 mM. Between protocols d and e the following steps were inserted to apply a test solution. (d1) A solution containing 1 mM EGTA and no Ca, and the other ionic constituents were similar to those of the test solution. Applied for 60 s. (d2) The test solution was applied for various lengths of time. The Ca$^{2+}$ concentration of the test solution was buffered with 10 mM EGTA. (d3) G10R to wash out the test solution. Applied for 60 s. After protocol i, the sequence of solution changes was repeated from d1 through i to estimate the reference level. This was necessary for the subtraction of both the Ca$^{2+}$-independent and "creep" components in the fluorescence signal (cf. Fig. 2).

10 s before each solution change, the flow of the solution was halted so that the tip of the tubing could be immersed in the next solution. At the time of a solution change, a new solution was flushed for 1 s into the capillary cuvette and the flow was restarted. However, in the case of ASSAY-F solution, only the flush was carried out, so that the solution around the fiber bundle was changed to ASSAY-F, but the Ca released would remain in the capillary.

Towards the end of the study, a 16-channel valve with a multiposition electric actuator (ECSD-16P, Valco Instruments Co. Inc., Houston, TX) was used for the selection of solutions to be led into the capillary cuvette. This valve was interfaced with the computer, and experiments could be conducted automatically following preprogrammed protocols.

With repetition of runs, a gradual rundown of the response was observed (diminution of $\sim$3–5% per run depending on the type of experiment). To allow for the rundown, we inserted an internal control for every two to five test runs.

At the end of each experiment, we measured the fluorescence intensity of ASSAY-F solution with 100 $\mu$M Ca added. This indicated the maximum fluorescence increase of fura-2 under that experimental condition. The fluorescence intensity of ASSAY-F with 1 mM EGTA added was also measured to determine the fura-2 fluorescence intensity without Ca. The difference in the fluorescence intensity with and without Ca was used for the calibration of the amount of Ca released from the skinned fiber bundle. The background fluorescence due to the capillary, immersion oil, and the fiber bundle was only a small percentage of the fluorescence intensity of 30–40 $\mu$M fura-2 without Ca in the capillary.

**Diffusion of Ca along the Capillary Cuvette during Assay**

Ca may diffuse away along the capillary cuvette. Although Ca concentration at the ends of the fiber bundle may rapidly decline, that near the center should change very slowly. Because only a 0.8-mm-long portion in the center of a 5-mm-long fiber bundle was illuminated for data collection, decrease in the Ca signal due to longitudinal diffusion should be minimal and was not taken into account in the analysis of data. This was supported by a calculation of diffusion of Ca, and there is indeed no sign of rapid decline in the fluorescence signals (e.g., Fig. 2).

**RESULTS**

**Extraction of Ca Signal from Fluorescence Intensity Change of Fura-2**

Fig. 2 A shows superimposed traces of the fluorescence intensity change, obtained from the same fiber bundle, upon application of solution ASSAY-F which contains
50 mM caffeine and 25 mM AMP but no ATP. AMP was added to the assay solution because adenine nucleotides enhance the caffeine-induced Ca release mechanism (see below). The uppermost trace (a) was obtained after Ca loading at pCa 6 in the presence of 4 mM MgATP for 144 s. There was a rapid step increase in the fluorescence intensity, followed by a creep. Two control runs are also shown. The lowermost trace (c) was obtained when no Ca loading procedure was carried out before the assay (no loading). The step size was smaller and the creep less steep. The middle trace (b) corresponds to the control run in which Ca loading was replaced by a Ca\(^{2+}\) treatment at pCa 6 for 144 s in the absence of ATP. The step size was same as that in "no loading". This small step increase in fluorescence intensity was mainly due to the direct effect of caffeine on fura-2 fluorescence, because similar increase was observed without the fiber bundle or with 380 nm excitation where the direction of Ca signal was opposite. The slope of the creep depended on both the duration of Ca loading and the Ca\(^{2+}\) concentration during the loading; the higher the Ca\(^{2+}\) concentration and/or the longer the duration, the steeper the creep irrespective of the presence or the absence of MgATP. Therefore, the creep seemed to be due mostly to slow liberation of Ca passively trapped by the preparation. Thus only the difference in the step size should correspond to the amount of Ca actively taken up by the store and released by the application of solution ASSAY-F.

To determine the step size objectively, we employed an extrapolation method. First, an average of the fluorescence intensity before Ca assay was subtracted from all the data points. Second, a straight line was fitted by a least-square method to the data points obtained between 20 and 60 s after the caffeine application. The line
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was extrapolated to the time of application of ASSAY-F (Fig. 2 A, dotted lines and circles) to obtain the step size. The difference in the step size with and without Ca loading was taken as the amount of Ca released from the preparation upon application of solution ASSAY.

An alternative method to extract the signal corresponding to Ca actively taken up by the fiber bundle and released with solution ASSAY-F is to plot the difference between records obtained in Ca loading with and without ATP, i.e., the upper trace of Fig. 2 A minus the middle trace (Fig. 2 B). The size of the Ca signal was determined by fitting a straight line to the plateau and extrapolating back to the time of the application of solution ASSAY-F to minimize the cancellation error of the creep. The both methods gave similar results. The extrapolation method was employed to obtain Fig. 3 A. The rest of the data were obtained using the alternative subtraction method.

**FIGURE 3.** Time course of Ca uptake by the store. Ca loading was carried out at pCa 7 (triangles), pCa 6 (circles), and pCa 5 (squares) for the period of time shown in the abscissa, and the amount of Ca taken up by the store was assayed by the application of caffeine (A) or both caffeine and IP$_3$ (B) in the presence of fura-2. Note that 1.0 in the ordinate in A should correspond to ~0.4 in B in terms of the absolute amount of Ca. Ca loading was carried out at 1.5 mM Mg$^{2+}$, 4 mM MgATP$^+$, and pH 7.0. Means of three to four experiments. Vertical lines indicate SEM.

*Time Course of Ca Loading to Caffeine- and Inositol 1,4,5-Trisphosphate-releasable Ca Store*

Inositol 1,4,5-trisphosphate (IP$_3$) has been postulated as an intracellular second messenger (Berridge and Irvine, 1984), and releases Ca from permeabilized smooth muscle cells (Suematsu et al., 1984; Somlyo et al., 1985, Yamamoto and van Breemen, 1985). It has been shown that there is a fraction of Ca store which cannot be released with caffeine, whereas simultaneous application of IP$_3$ and caffeine releases all the Ca from skinned fiber bundles of taenia (Iino, 1987).

To determine the time course of the Ca uptake by the store, skinned fiber bundles were treated with Ca$^{2+}$ (pCa 5, 6, and 7) for various lengths of time (15–720 s) and the amount of loaded Ca was assayed afterward by the application of 50 mM caf-
feine, solution ASSAY (Fig. 3 A) or by the simultaneous application of 50 mM caffeine and 10 μM IP3, solution ASSAY supplemented with 10 μM IP3 (Fig. 3 B) in the presence of fura-2. Under the respective assay condition the size of the Ca response, after a Ca loading at pCa 6 for 120 s was used as an internal standard to normalize the responses. Note that the absolute amount of the standard for caffeine + IP3-releasable store was about twice as large as that for caffeine alone (Iino, 1987).

At pCa 7, Ca was accumulated in the caffeine-releasable store slowly, taking 12 min or more to reach plateau. At pCa 6, the initial rate of the Ca uptake was about eight times faster than that at pCa 7, and the plateau level was nearly twice as large. The time courses of the Ca uptake by the total store at pCa 6 and 7 are similar to that by caffeine-releasable store, if normalized. The Ca uptake by caffeine-releasable store at pCa 5 reaches plateau, which is ~40% of that at pCa 6, rapidly with a half-time of <15 s. On the other hand, total Ca store accumulates Ca at pCa 5 more slowly with a half-time of some 45 s, and the plateau level is ~60% of that at pCa 6. The initial speed of Ca uptake, however, is similar both at pCa 6 and 5 in either assay method. The steady level with pCa 5 was smaller than that with pCa 7 in the caffeine-releasable store, but this relation was reversed in the total store.

Absolute Amount of Releasable Ca in the Store

To estimate the absolute capacity of the Ca store the following factors were taken into account (Iino, 1987). (a) The size of the Ca signal normalized to the maximum fluorescence signal of fura-2 with saturating Ca in the solution ASSAY-F. (b) Volume ratio of the fiber bundle to the free space in the capillary cuvette. The volume of the bundle was estimated by the widths measured at two directions at ~90° assuming an ellipsoidal cross-section. (c) Volume ratio of cell water space to the volume of the fiber bundle. This was assumed to be 0.5, for the extracellular space of taenia occupies 32% of the total volume (Gabella, 1976) and the ratio of dry weight to wet weight is reported to be 0.17 (Axelsson and Holmberg, 1971). (d) The leakage of Ca from the store during the 4-min interval between the end of Ca loading and the moment of Ca assay.

After the previous report (Iino, 1987), Ca leakage rate has been slightly revised (0.2 min⁻¹, see below) and the number of measurement has been increased. The current value for the amount of Ca released with caffeine + IP3 from the store after Ca loading at pCa 6, 1.5 mM Mg²⁺ for 120 s (1.0 in the ordinate in Fig. 3 B) is 149 ± 30 μmol/liter cell water (mean ± SD, n = 6). Therefore, the maximum capacity of the Ca store, which is ~1.5 times this value (Fig. 3 B), is enough to increase total Ca concentration in the cell by ~220 μM.

Ca-induced Ca Release Mechanism in the Caffeine-releasable Store

Fig. 3 A shows that steady-state amount of releasable Ca in the caffeine-releasable store declines above an optimal Ca²⁺ concentration. Similar biphasic dependence of Ca uptake upon Ca²⁺ concentration in caffeine-releasable store has been obtained in skinned smooth muscle fiber bundles using a caffeine contracture method (Itoh et al., 1981; Saida, 1982). One of the explanations for this phenomenon is the operation of a Ca-induced Ca release mechanism at higher Ca²⁺ concentrations, thus making the balance between Ca uptake and release favor the release. However,
there could be other possibilities, such as inhibition of Ca pump ATPase or formation of insoluble Ca precipitate within the store at higher Ca\(^{2+}\) concentrations, albeit the former is less likely because the initial speed of Ca uptake is not inhibited at pCa 5 (Fig. 3).

To test whether there is indeed a CICR mechanism in the caffeine-releasable store, we applied Ca\(^{2+}\) to the store in the absence of ATP and the release of Ca was examined. For a quantitative measurement of the CICR, the Ca\(^{2+}\) concentration during the Ca release has to be clamped using a high concentration of EGTA, but under such a condition it is impossible to directly measure the amount of released Ca using the present fluorescent dye method. An alternative method is to load the store with a fixed amount of Ca, then to apply well-buffered test Ca\(^{2+}\) in the absence of ATP, and finally to assay the amount of remaining Ca in the store (for the detail of the protocol see Methods). The decline in the remaining Ca as compared with that without a test procedure should be proportional to the amount of Ca released during the test Ca\(^{2+}\) application.

**Figure 4.** Time course of the Ca release from caffeine-releasable Ca store. Starting from a fixed amount of Ca in the store, fiber bundles were treated with 10 mM EGTA (open squares) or with pCa 5.7 (solid circles) in the absence of both Mg\(^{2+}\) and ATP for the length of time indicated on the abscissa. Ca remaining in the store after the treatment is plotted, pH 7.0. Solutions: RMg0. Means and SEM of three experiments in each condition.

Fig. 4 shows the results of such experiments. Test solutions buffered with 10 mM total EGTA either at pCa > 8 (open squares) or at pCa 5.7 (solid circles) were applied in the absence of Mg\(^{2+}\) and ATP for the length of time indicated on the abscissa. The amount of remaining Ca in the caffeine-releasable store was normalized by that of the control run without a test procedure. There was a gradual decrease of Ca in the store in the virtual absence of Ca\(^{2+}\) with a half-time of ~6 min. Similar rate has been found for the Ca leakage from the sarcoplasmic reticulum of the extensor digitorum longus muscle in the guinea pig (Ohta, T., personal communication). A significant feature in Fig. 4 is that the rate of decline of Ca in the store was definitely enhanced by the rise in the Ca\(^{2+}\) concentration to pCa 5.7. This clearly shows that there is indeed a CICR mechanism in the caffeine-releasable Ca store. A condition which would activate the CICR mechanism noticeably but not extensively was chosen in Fig. 4 to show the full time course of the Ca release. The rate of the CICR can be much greater as shown below.
It can be also seen in Fig. 4 that the time course of the Ca release is nearly exponential and approaches zero. Therefore, in the following experiments, the duration of each test application was chosen so that the remaining Ca was about one to two thirds of the control and the activity of the CICR is expressed by the rate constants, assuming an exponential decline.

**Dependence of the Ca-induced Ca Release Rate on Ca, pH, Adenine Nucleotide, and Mg**

The rates of the CICR in the absence of Mg\(^{2+}\) and at pH 7.0 are plotted against pCa by the circles in Fig. 5. As Ca\(^{2+}\) concentration exceeds pCa 6, the rate of Ca release becomes greater than the Ca leakage rate. At the foot of the curve the increase in the rate is nearly proportional to the square of the Ca\(^{2+}\) concentration, whereas at higher Ca\(^{2+}\) concentrations the slope of the curve becomes less steep than expected for a parabolic dependence.

The rate of Ca release was determined at various total EGTA concentrations to see if the Ca buffer had any direct effect on the Ca release mechanism. Ca release rate at pCa 5.5 was 0.885 ± 0.129, 0.852 ± 0.116, 0.946 ± 0.112, and 1.048 ± 0.164 (mean ± SEM, n = 6) at 2, 5, 10, and 20 mM EGTA\(_{\text{total}}\), respectively. The differences were not statistically significant (T test).

Rates of the CICR were also measured at both pH 6.7 and 7.3, and compared with those at pH 7.0 (Fig. 5). With a higher H\(^{+}\) concentration a higher Ca\(^{2+}\) concentration was required to activate the CICR mechanism to the same level, and a change in pH by 0.3 shifted the curve ~0.5 pCa units.

The CICR mechanism in striated muscle is enhanced by adenine nucleotides (Endo, 1977, 1985). Whether this is also true in smooth muscle was tested by studying the effect of AMPOPCP, a nonhydrolyzable ATP analogue on the rate of the CICR at pCa 5.5 in the presence of 1.5 mM Mg\(^{2+}\). The rate increased in a nearly hyperbolic manner with the AMPOPCP concentration. The rates of the CICR were 0.44, 0.71, and 0.83 min\(^{-1}\) with 0.3, 1, and 3 mM AMPOPCP, respectively (means of data from three fiber bundles). Therefore, the AMPOPCP concentration for the half maximum response is near 0.3 mM.

pCa dependence of the rate of the CICR mechanism in the presence of 2 mM AMPOPCP, which produces a nearly maximum potentiating effect on the Ca...
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Release mechanism, was compared with that obtained in the absence of the ATP analogue. As shown by the triangles and the open circles in Fig. 6, 1.5 mM Mg$^{2+}$ shifts the relation between Ca$^{2+}$ concentration and the rate of CICR toward the higher Ca$^{2+}$ concentration in the absence of the nucleotide. With addition of 2 mM AMPOPCP at 1.5 mM Mg$^{2+}$, the rates of the Ca-induced Ca release became greater at Ca$^{2+}$ concentrations above 1 μM (Fig. 6, solid circles). However, the range of the Ca$^{2+}$ concentrations in which the rate of the Ca release changed was nearly the same with and without AMPOPCP.

Results shown in Fig. 6 show that the rate of the CICR is dependent on the Mg$^{2+}$ concentration. The effect of Mg$^{2+}$ was further studied in the presence of 2 mM AMPOPCP, and the results are shown in Fig. 7. Mg$^{2+}$ decreased the Ca sensitivity of the CICR mechanism, and a threefold increase in Mg$^{2+}$ concentration shifted the curve nearly 0.5 pCa unit to the right.

Effect of Caffeine and Procaine on the CICR

Caffeine is known to enhance the CICR mechanism in skeletal muscle. This ability to enhance the Ca release mechanism is responsible for the Ca releasing action of the drug in striated muscles (Endo, 1977, 1985). By analogy, the Ca releasing action of
the drug in smooth muscle has been attributed to the enhancement of the CICR. One can now directly test this notion by including caffeine during the test procedure in experiments to study the Ca release mechanism. Thus, if 5 mM caffeine was added to the test solutions in the presence of 1.5 mM Mg\(^{2+}\) and 2 mM AMPOPCP, the rate of the Ca release was very much enhanced (Fig. 7, solid circles). At pCa 6 or 7, where no detectable CICR is observed without the drug, there is a clear enhancement of the Ca release. The most striking effect of caffeine was to make the CICR mechanism ~30 times more sensitive to Ca\(^{2+}\). However, the effect of the drug was hardly seen in the absence of Ca\(^{2+}\). This indicates that the drug itself has no direct Ca-releasing action.

The effect of caffeine was dose dependent. The rates of the CICR at pCa 7, 1.5 mM Mg\(^{2+}\), 2 mM AMPOPCP, pH 7 in the presence of 0, 1, 5, and 25 mM caffeine were 0.075 ± 0.019, 0.154 ± 0.022, 0.629 ± 0.094, and 4.83 ± 0.36 min\(^{-1}\) (Mean ± SEM, n = 6), respectively. There was a marginal increase in the rate of Ca release at 1 mM caffeine (P < 0.05, T test), and a definite increase above 5 mM (P < 0.01).

Procaine has an inhibitory effect on the CICR mechanism of striated muscle (Endo, 1977) in addition to its well-known effect on the sodium channels. This drug also inhibited the smooth muscle CICR mechanism in a dose-dependent manner. The rate of the CICR at pCa 5.5, 1.5 mM Mg\(^{2+}\), 2 mM AMPOPCP, and pH 7.0 was compared at various procaine concentrations. Means of three measurements were 0.63, 0.48, 0.21, and 0.12 min\(^{-1}\), with 0, 1, 3, and 10 mM procaine, respectively. Although the dose-response relation was not a simple hyperbolic one, ~2 mM procaine was required for half-maximum inhibition.

Absence of the CICR Mechanism in the Caffeine-insensitive Store

Results so far suggest that caffeine releases Ca from only a fraction of the store through activation of the CICR. The rest of the Ca store does not respond to caffeine, and hence it is likely to be devoid of the CICR mechanism. The absence of the CICR mechanism in the caffeine-insensitive store was further examined in the following experiments.

The amount of Ca in the caffeine-releasable store declines nearly exponentially to zero if the CICR is activated by Ca\(^{2+}\) in the absence of ATP (Fig. 4). However, if only a fraction of the Ca store has the Ca-induced Ca release mechanism, such exponential decline to zero can no longer be expected when the total Ca store was examined with caffeine + IP\(_3\) assay.

In Fig. 8 the CICR was activated during the test procedure at pCa 6.5 with an enhancer 10 mM caffeine for the duration shown in the abscissa, and the amount of Ca remaining in the caffeine-releasable store (open symbols) was compared with that in the caffeine + IP\(_3\)-releasable store (solid symbols). 1.0 in the ordinate corresponds to the amount of Ca released by caffeine + IP\(_3\) assay without the Ca releasing test procedure. Open symbols indicate that the amount of Ca in the caffeine-releasable store rapidly declines to zero under the experimental condition. The amount of Ca in the total store (solid symbols) declined in two phases, i.e., an initial rapid decline, whose magnitude and time course were very similar to those of the caffeine assay, and a late slow decline. In a similar experiment but with a different condition for
the Ca-induced Ca release activation (pCa 5.7), the amount of Ca in the caffeine + IP₃-sensitive store also declined in two phases, giving rise to a kink at the similar height. These results are consistent with the notion that only a fraction (~40%) of the store has the Ca-induced Ca release mechanism.

The above results predict that a part of the Ca store which does not have the Ca-induced Ca release mechanism may be able to take up Ca even in the presence of a high concentration of caffeine, a strong potentiator of the Ca release mechanism. This was tested in the following experiments. First, Ca in the store was depleted by the caffeine + IP₃ assay. Then Ca loading was carried out at pCa 6 for 120 s in the absence (control) or presence of 50 mM caffeine. After such Ca loading in caffeine, Ca release could be barely observed with caffeine assay, whereas ~55% of the control response could be observed when the assay solution contained IP₃.

**FIGURE 8.** Comparison of the time course of the Ca-induced Ca release from the total Ca store and that from the caffeine-releasable store. Starting from a fixed amount of Ca in the store, fiber bundles were treated with a test solution containing Ca²⁺ at pCa 6.5, no Mg²⁺, and 10 mM caffeine for the period of time in the abscissa. Ca remaining in the store was assayed either by caffeine (open symbols) or by caffeine + IP₃ (solid symbols). Results obtained from two experiments.

**DISCUSSION**

The present experiments demonstrate that the CICR mechanism in smooth muscle shares common properties with the skeletal muscle CICR mechanism (Endo, 1977, 1985) and also with the high-conductance Ca channel of both heavy SR vesicles and purified ryanodine receptors incorporated into planar bilayers (Smith et al., 1986; Rousseau et al., 1986, 1988; Imagawa et al, 1987; Hymel et al., 1988; Lai et al., 1988) in that the activity of the mechanism is dependent on micromolar concentration of Ca²⁺, millimolar concentrations of adenine nucleotides, Mg²⁺ and caffeine, and pH. It has been shown that treatment of skinned fiber bundles of smooth muscle with ryanodine depletes caffeine-sensitive store due to open lock of the CICR channels with the drug, while leaving the rest of the store intact (Iino et al., 1988). These findings suggest that the CICR channels in smooth muscle are equivalent to skeletal muscle ryanodine receptors.

Skeletal muscle ryanodine receptors seem to form junctional feet which span between the T-tubule and the SR (Inui et al., 1987; Lai et al., 1988). Similar bridging structures, although less well developed than skeletal muscle junctional feet,
have been identified between the surface membrane and the SR of smooth muscle by electron microscopy (Devine et al., 1972). Thus, it is an interesting possibility that the bridges correspond to the CICR channels in smooth muscle.

To avoid confusion in terminology, it is pertinent to state here that the CICR mechanism referred to in this paper is different from "time-dependent Ca-induced release of Ca" described only in cardiac muscle so far (Fabiato, 1985). Either the CICR mechanism originally characterized in skeletal muscle (Endo, 1977, 1985) or the SR Ca-release channels incorporated into bilayer membranes (Smith et al., 1986) does not show apparent inactivation. Neither does the CICR mechanism of the present study show obvious inactivation process. It remains to be seen if a time-dependent or rapidly inactivating Ca release mechanism such as that of Fabiato is present in smooth muscle. However, slowness of smooth muscle Ca transients (Morgan and Morgan, 1984; Himpens and Somlyo, 1988) might limit the role of rapidly inactivating Ca release mechanisms.

Comparison with the Previous Skinned Fiber Methods

Ca release from the intracellular Ca store in skinned smooth muscle fiber bundles was first detected by Endo and his colleagues (Endo et al., 1977), and later by other authors (Itoh et al., 1981; Saida, 1982). These authors have assayed the Ca released from the store using the contractile system of the preparation as an intrinsic Ca indicator, i.e., they measured the magnitude of transient tension development after the release of Ca from the store. The present method to use fura-2 for Ca measurement is more appropriate for quantitative analysis in the following points. (a) Reproducibility. Because the Ca sensitivity of the contractile system of smooth muscle skinned fibers declines rapidly with repeated contractions (Endo et al., 1977; Iino, 1981), it has been difficult to carry out quantitative study using the contractile system as a Ca indicator. The present study circumvented this problem by the use of a fluorescent Ca indicator to assay the amount of Ca released from the store. (b) Linearity. The present method has an inherently linear relation between the change in the fluorescence intensity and the amount of Ca released. This has not been tested vigorously in the previous skinned smooth muscle fiber experiments. (c) Buffering capacity of Ca\(^{2+}\). Ca\(^{2+}\) concentrations were strongly buffered with 10 mM EGTA during test procedures in this study. Previous studies have usually used 0.1 mM EGTA, and the results could have been affected by the poor buffering capacity.

Radioactive Ca was used to measure the amount of Ca taken up by skinned smooth muscle strips or skinned cultured vascular smooth muscle cells (Stout and Diecke, 1983; Yamamoto and van Breemen, 1985). Usually only one measurement can be carried out for one sample with the \(^{45}\)Ca activity. Ca assays can be repeated several times on the same fiber bundle by the fluorescence method used in this work.

Heterogeneity and Ca Uptake Capacity of the Store

The amount of Ca taken up by the caffeine-releasable store at pCa 5 was smaller than that at pCa 7 (Fig. 3 A), and this can be explained by the operation of the CICR at the higher Ca\(^{2+}\) concentration. However, if the total Ca store was exam-
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In my previous report, the IP₃ assay solution released ~10% less Ca from the store than the IP₃ + caffeine assay solution did (Fig. 1, d vs. f, of Iino, 1987). Since then, it has been shown that the IP₃-induced Ca release is enhanced by adenine nucleotides. Therefore, if 25 mM AMP had been added to the IP₃ assay solution, it would have released almost the same amount of Ca as the IP₃ + caffeine assay solution, which contained AMP. In fact this was proved the case (unpublished observation). Therefore, there seems to be little, if any, compartment which has the CICR mechanism and lacks the IP₃-induced Ca release mechanism.

It is an important question how the two components of the Ca store are attributed to subcellular structures. Mitochondria is an unlikely candidate for either compartment, because the both components accumulate Ca at pCa 7, whereas mitochondria of skinned fiber bundles have very little Ca content even at pCa 6 (Somlyo et al., 1982), and because the experiments were carried out in the continued presence of sodium azide, a mitochondrial uncoupler. Somlyo and co-workers have shown, using the electron probe x-ray microanalysis, that both junctional and central SR accumulate Ca and release Ca upon agonist stimulation in rabbit main pulmonary artery (Kowarski et al., 1985). It is tempting to assume that the two compartments correspond to the SR of different location. However, one has to take this possibility with caution, because the junctional and central SR are structurally continuous (Devine et al., 1972). It is not known whether the two structures can be functionally discontinuous. One of the possibilities that has not been tested is the heterogeneity of cells. It is conceivable that a population of the smooth muscle cells have a single class of Ca store with both CICR and IP₃-induced Ca release mechanism, whereas the rest of the cells have the Ca store with only IP₃-induced Ca release mechanism. Elucidation of this issue requires further study.

The maximum amount of Ca taken up in the smooth muscle Ca store is some 220 μmol/liter cell water. If the structure responsible for the Ca uptake capacity is the SR, Ca concentration within the SR would be as high as 15 mmol/liter because the volume occupied by the SR is only ~1.5% in taenia (Devine et al., 1972). At pCa 7, which roughly corresponds to the Ca²⁺ concentration of relaxed muscle cells, the Ca content of the SR would be about a half of the maximum value. This value is comparable to the amount of Ca measured by electron probe x-ray microanalysis in the SR of guinea pig portal vein (28 mmol/kg dry weight, Bond et al., 1984) and that in the central SR of rabbit main pulmonary artery (42–49 mmol/kg dry weight, Kowarski et al., 1985), if a factor of 3 to 4 is taken into account for the ratio of wet weight to dry weight.

Rates of the CICR under a Physiological Condition and Its Significance

Present study demonstrates the first direct evidence for the presence of the CICR mechanism in smooth muscle and provides its quantitative properties. pH, Mg²⁺, and adenine nucleotides are found to be the important physiological modulators of the CICR. One needs to know the physiological condition of these parameters to estimate the rate of CICR in living cells. Values reported for intracellular pH
deduced from the $^{31}$P-nuclear magnetic resonance (NMR) studies of various smooth muscle tissues lie between 7.0 and 7.1 (Hellstrand and Vogel, 1985; Kushmerick et al., 1986).

Intracellular Mg$^{2+}$ concentration in rabbit bladder and uterine smooth muscle has been estimated by Kushmerick et al. (1986) by the chemical shifts of the nucleotides in $^{31}$P NMR spectroscopy. Estimates thus derived are 0.40 mM for the intracellular Mg$^{2+}$ concentration in uterus, and 0.46 mM for bladder. Mg$^{2+}$ concentrations were estimated from the calculation of multiequilibrium equations both in the calibration experiments for the NMR study as well as in the present study. If the difference in the binding constants used was allowed for, 0.4 mM in their scale would correspond to 0.93 mM in the present Mg$^{2+}$ concentration scale. Inversely, 0.5 and 1.5 mM in the present study would correspond to 0.23 and 1.3 mM, respectively, in the Mg$^{2+}$ concentration scale of the NMR study.

Recent chemical analyses of ATP in various smooth muscles indicate that ATP content is 0.6–1.2 μmol/g wet weight (Hellstrand and Vogel, 1985; Kushmerick et al., 1986). If the extracellular space and the dry weight to wet weight ratio of smooth muscle tissues are considered, these values correspond to ~1–2 mM, which is sufficiently high to exert almost the maximum effect on the CICR. But ambiguity remains as to the adenine nucleotide effect, because one could not use ATP in the experiment of the present experimental protocol, and the potency could not be directly compared between ATP and AMPOPCP. In spite of this uncertainty, the main effect of the adenine nucleotide on the CICR was a proportional increase of Ca release rates at all Ca$^{2+}$ concentrations >100 nM, and the nucleotide did not greatly change the range of effective Ca$^{2+}$ concentration as Mg$^{2+}$ or caffeine did.

In the light of the above considerations, the most plausible estimates for the rates of CICR under a physiological condition lie between the values obtained at 0.5 and 1.5 mM Mg$^{2+}$, 2 mM AMPOPCP, and pH 7 (Fig. 7, squares and open circles). The slope of the curve might be steeper if ATP was indeed more potent than its analogue, AMPOPCP. A general conclusion drawn from these results is that it is not until the intracellular Ca$^{2+}$ concentration exceeds 1 μM that the CICR mechanism becomes activated.

The relation between the Ca$^{2+}$ concentration and the steady developed tension of taenia caeci has been studied using similar skinned fiber bundles (Iino, 1981). Tension started to develop just above 100 nM Ca$^{2+}$ and reached maximum at 3–10 μM. Therefore, the intracellular Ca$^{2+}$ concentration in fully relaxed taenia seems near or less than 100 nM. At 1 μM Ca$^{2+}$, which is barely enough to activate the CICR mechanism under the physiological condition estimated above, developed tension is as high as 50–60% of the maximum tension. Thus, it is difficult to assume that the CICR mechanism plays a primary role in triggering a physiological contraction of taenia.

However, the present results do not completely exclude a physiological role of the CICR channels. Firstly, it is possible that the Ca$^{2+}$ concentration in the vicinity of the Ca store becomes locally and transiently high enough for a massive Ca release after a stimulation, although this requires a certain elaborate structure which forms an effective compartment adjacent to the Ca-releasing surface of the Ca store. Secondly, there could be unknown intrinsic factors which potentiate the CICR mecha-
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nism like caffeine does. Such factors would have been lost from skinned fiber bundles if they were soluble, and the present estimate of the Ca\(^{2+}\) release rate and its Ca\(^{2+}\) dependence could have been rather low. It is also possible that the artificial constituents of the experimental solutions had some adverse effect. Thirdly, it is conceivable that CICR channels may operate in a different, still unknown mode in response to a physiological stimulus, whereas Ca\(^{2+}\) has only a modulating effect. Further study is required to settle this point.

The present study demonstrates enhancement of the CICR by caffeine, and this effect seems to provide the basis for the caffeine-induced contractions of intact smooth muscle bundles. 5 mM caffeine had almost no Ca-releasing action in the absence of Ca\(^{2+}\), but it increased the rate of CICR eightfold at a near resting Ca\(^{2+}\) concentration of pCa 7 in the quasiphysiological condition. 1 mM of the drug also showed small twofold increase in the rate of CICR at pCa 7. In intact cells in a rested state, the rate of Ca release from the store is expected to be balanced with the rate of Ca uptake, and an enhancement of the CICR above resting value should result in a rise in the intracellular Ca\(^{2+}\) concentration, which would further increase the rate of the Ca release. Thus, after the application of caffeine, the intracellular Ca\(^{2+}\) concentration should increase until the rate of Ca sequestration overcomes the rate of Ca release. It is difficult to estimate the peak Ca\(^{2+}\) level thus attained without knowing the concentration and the kinetics of the intracellular Ca binding sites as well as the Ca\(^{2+}\) dependence of Ca extrusion mechanisms in both the Ca store and the plasma membrane. However, the results obtained in skinned fibers seem to be in accordance with the finding that the minimum concentration of caffeine for a contraction of intact taenia caeci is 1–5 mM at ~20°C (Ito and Kuriyama, 1971; Yagi et al., 1985).

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