In smooth muscle, release via the inositol 1,4,5-trisphosphate (Ins(1,4,5)P$_3$) and ryanodine receptors (RyR) on the sarcoplasmic reticulum (SR) controls oscillatory and steady-state cytosolic Ca$^{2+}$ concentrations ([Ca$^{2+}$]$_c$). The interplay between the two receptors, itself determined by their organization on the SR, establishes the time course and spatial arrangement of the Ca$^{2+}$ signal. Whether or not the receptors are co-localized or distanced from each other on the same store or whether they exist on separate stores will significantly affect the Ca$^{2+}$ signal produced by the SR. To date these matters remain unresolved. The functional arrangement of the RyR and Ins(1,4,5)P$_3$R on the SR has now been examined in isolated single voltage-clamped colonic myocytes. Depletion of the ryanodine-sensitive store, by repeated application of caffeine, in the presence of ryanodine, abolished the response to Ins(1,4,5)P$_3$, suggesting that Ins(1,4,5)P$_3$R and RyR share a common Ca$^{2+}$ store. Ca$^{2+}$ release from the Ins(1,4,5)P$_3$R did not activate Ca$^{2+}$-induced Ca$^{2+}$ release at the RyR. Depletion of the Ins(1,4,5)P$_3$-sensitive store, by the removal of external Ca$^{2+}$, on the other hand, caused only a small decrease (∼26%) in caffeine-evoked Ca$^{2+}$ transients, suggesting that not all RyR exist on the common store shared with Ins(1,4,5)P$_3$R. Dependence of the stores on external Ca$^{2+}$ for replenishment also differed; removal of external Ca$^{2+}$ depleted the Ins(1,4,5)P$_3$-sensitive store but caused only a slight reduction in caffeine-evoked transients mediated at RyR. Different mechanisms are presumably responsible for the refilling of each store. Refilling of both Ins(1,4,5)P$_3$-sensitive and caffeine-sensitive Ca$^{2+}$ stores was inhibited by each of the SR Ca$^{2+}$ ATPase inhibitors thapsigargin and cyclopiazonic acid. These results may be explained by the existence of two functionally distinct Ca$^{2+}$ stores; the first expressing only RyR and RyR and dependent upon external Ca$^{2+}$ for refilling.

Release of Ca$^{2+}$ from the sarcoplasmic reticulum (SR) store,

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§ A Caledonian Research Foundation Fellow. To whom correspondence should be addressed: Tel.: 44-141-330-5143; Fax: 44-141-330-6610; E-mail: j.mccarron@bio.gla.ac.uk.

The abbreviations used are: SR, sarcoplasmic reticulum; Ins(1,4,5)P$_3$, inositol 1,4,5-trisphosphate; Ins(1,4,5)P$_3$R, Ins(1,4,5)P$_3$ receptor; RyR, ryanodine receptor; CICR, Ca$^{2+}$-induced Ca$^{2+}$ release; [Ca$^{2+}$]$_c$, cytosolic Ca$^{2+}$ concentration; SERCA, sarcoplasmic reticulum Ca$^{2+}$ ATPase; FF, the ratio of fluorescence counts (F) relative to baseline counts before stimulation (F$_o$); ΔF/F$_o$, the magnitude of the change in F/F$_o$; IBMX, 3-isobutyl-1-methylxanthine; [cAMP], cytosolic concentration of cAMP; CPA, cyclopiazonic acid.
nder smooth muscle cells (23) of three Ca^{2+} stores, one containing RyR and Ins(1,4,5)P_{3}R, another expressing only Ins(1,4,5)P_{3}R, and a third containing only RyR.

Whereas the proposed arrangements of RyR and Ins(1,4,5)P_{3}R may reflect the complexity of the underlying biology, differences in experimental approaches may also have contributed to the variety of views expressed. For example, caffeine, commonly used to activate RyR, also inhibits Ins(1,4,5)P_{3}R (24, 25). In some studies (e.g. 12, 21) caffeine remained present while an Ins(1,4,5)P_{3}-generating agonist was applied. Invariably, such experiments demonstrated an inhibition of the Ins(1,4,5)P_{3}-mediated response and the results have been taken as evidence for the existence of a common Ca^{2+} store. Inhibition of the Ins(1,4,5)P_{3} receptor by caffeine, rather than depletion of a common store, could have accounted for the absence of response to an Ins(1,4,5)P_{3}-generating agonist.

Additional difficulties in the classification of SR Ca^{2+} stores, *i.e.* their location and number, have followed the use of plasmalemmal agonists and the multiple, yet separate, biochemical pathways so activated. Two particular aspects of such difficulties are evident. First, when membrane currents are used as indicators of [Ca^{2+}]_{e}, agonists may modify these currents independently of SR Ca^{2+} release (e.g. 28, 29). Second, regulation of the RyR and Ins(1,4,5)P_{3}R by Ca^{2+} derived from agonist activation of several different biochemical pathways may occur with misleading consequences. For example, in rabbit portal vein, depletion of the Ins(1,4,5)P_{3}-sensitive store by norepinephrine, abolished the response to caffeine (which acts on the RyR (27)), consistent with both receptors residing on a common Ca^{2+} store. On the other hand, Ca^{2+} released from the SR via Ins(1,4,5)P_{3}R activation may have triggered a regenerative Ca^{2+}-induced Ca^{2+} release (CICR) at the RyR (11, 28), which could have amplified the Ins(1,4,5)P_{3}-evoked Ca^{2+} transient. If so, two outcomes could be anticipated (a) the continued presence of Ins(1,4,5)P_{3} could deplete the RyR-sensitive Ca^{2+} pool; (b) depletion of the RyR-sensitive Ca^{2+} pool would reduce the response to Ins(1,4,5)P_{3}. Either of these results could be misinterpreted as support for the existence of a common Ca^{2+} store.

Notwithstanding these difficulties, it is important to determine the arrangement of Ca^{2+} stores in smooth muscle to help clarify the precise mechanisms of Ca^{2+} release, a vital ingredient in our understanding of contractility. This problem has been addressed in the current investigation by seeking answers to the following questions: 1) Are Ins(1,4,5)P_{3}R and RyR present on the same store or on separate stores of the SR? 2) Does Ca^{2+} released from the Ins(1,4,5)P_{3}-sensitive store trigger CICR via activation of the RyR? 3) Are there differences between the refilling mechanisms of Ins(1,4,5)P_{3}-sensitive and ryanodine-sensitive intracellular Ca^{2+} stores? In this study freshly isolated single smooth muscle cells rather than multi-cellular preparations were used, removing the difficulty of there being different store characteristics existing in different cells or of store reorganization, which may accompany the use of cell culture preparations. Ca^{2+} influx was controlled under voltage clamp conditions and directly measured in this investigation. Flash photolysis of caged Ins(1,4,5)P_{3} (Ins(1,4,5)P_{3}) and caffeine were each used to minimize activation of second messenger systems so that a clearer understanding of the control of Ca^{2+} release from the receptors could be obtained. From the results of the present study it is proposed that two functionally distinct SR Ca^{2+} stores exist in colonic myocytes; one expressing both Ins(1,4,5)P_{3}R and RyR and dependent upon an external Ca^{2+} source for replenishment and a second store containing only RyR, which can be refilled form [Ca^{2+}]_{e}.

**EXPERIMENTAL PROCEDURES**

**Cell Isolation**—From male guinea pigs (500–700 g) stunned by a blow to the head and immediately killed by exsanguination, a segment of distal colon (~5 cm) was removed. The circular muscle was separated from the longitudinal layer, and single cells were prepared from the former using a two-step enzymatic process (30), stored at 4 °C, and used the same day.

**Membrane Current Recording**—Cells were voltage-clamped in the dialyzed whole cell configuration. Currents were amplified by an Axopatch 1D (Axon Instruments, Union City, CA), low-pass filtered at 500 Hz (8-pole Bessel filter, Frequency Devices, Haverhill, MA), and digitally sampled at 1.5 kHz using a Digidata interface. pCLAMP software (version 6.0.1, Axon Instruments), and Axotape (Axon Instruments) and stored for analysis. Cells were held at a membrane potential (V_{m}) of ~70 mV unless otherwise indicated. The bathing solution contained (mM): sodium glutamate, 80; NaCl, 40; tetraethylammonium chloride, 20; MgCl_{2}, 1.1; CaCl_{2}, 3; HEPES, 10; and n-glucose, 30; pH 7.4 adjusted with NaOH. The Ca^{2+}-free bathing solution contained MgCl_{2} (3 mM) and EGTA (1 mM). The pipette solution contained (mM): CsSO_{4}, 85; CsCl, 20; MgCl_{2}, 1; MgATP, 3; pyruvic acid, 2.5; malic acid, 2.5; NaH_{2}PO_{4}, 1; creatine phosphate, 5; GTP, 0.5; HEPES, 30; fluo-3-penta-ammonium salt, 0.1; caged Ins(1,4,5)P_{3}, 0.025; pH 7.2 adjusted with CsOH. The access afforded by the whole cell patch electrode allowed entry into the cell of the membrane-impermeant fluo-3 and caged Ins(1,4,5)P_{3}.

**Cytosolic Ca^{2+} Concentration ([Ca^{2+}]_{c}) Measurement—**[Ca^{2+}]_{c}, was measured via the membrane-impermeable fluo-3 (penta-ammonium salt). Cytosolic fluorescence measurements were made using a microfluorometer consisting of an inverted fluorescence microscope (Nikon Diaphot) and a photomultiplier tube with a bi-alkali photocathode. Fluo-3 was excited at 488 nm (bandpass 9 nm) from a PFI Delta Scan (Photon Technology International Inc., East Sheen, London, UK) through the epi-illumination port of the microscope (using one arm of a bifurcated quartz fiber optic bundle). Excitation light was passed through a field stop diaphragm, to reduce background fluorescence, and reflected off a 505-nm long-pass dichroic mirror; emitted light was guided through a 555-nm barrier filter (bandpass 35 nm) to a photomultiplier in photon-counting mode. Longer wavelengths, from bright field illumination with a 610-nm Shott glass filter, were reflected onto a charge-coupled device camera (Sony model XC-75) mounted onto the viewing port of the Delta Scan thus allowing the cell to be monitored during experiments. Interference filters and dichroic mirrors were obtained from Glen Spectra (London, UK). To photolyse caged Ins(1,4,5)P_{3}, the output of a xenon flashlamp (Rapp OptoElektron, Hamburg, Germany) was passed through a UG-5 filter to select ultraviolet light and merged into the excitation light path of the microfluorometer using the second arm of the quartz bifurcated fiber optic bundle. The nominal flash lamp energy was 57 mJ, measured at the output of the fiber optic bundle. The flash duration was about 1 ms. Caffeine (10 mM) was applied by hydrostatic pressure (Pneumatic PicoPump PV820, World Precision Instruments, Inc., Sarasota, FL). All experiments were carried out at room temperature (18–22 °C), and drugs were applied either hydrostatically via a pipette or into the bathing solution as indicated in the text.

**Data Analysis**—Changes in cytosolic Ca^{2+} were expressed as a ratio (F/F_{0}) of the fluorescence counts (F) relative to baseline counts before stimulation (F_{0}). ΔF/F_{0} indicates the magnitude of the change in F/F_{0} at the peak of the evoked transient relative to the baseline ratio. Original fluorescence records were not filtered, smoothed, or averaged. Background fluorescence was not subtracted. Statistical analyses were performed using either Mann-Whitney tests (on normalized data) or paired Student’s t-tests (on raw data). Summarized data are shown as means ± S.E. and taken to be statistically significant when p < 0.05. n indicates numbers of cells used.

**Drugs and Reagents—**fluo-3 penta-ammonium salt was obtained from Molecular Probes, Inc. (Eugene, OR). Caged Ins(1,4,5)P_{3}, trisodium salt, thapsigargin, cyclopiazonic acid (CPA), and ryanodine were obtained from Calbiochem-Novabiochem Ltd. Thapsigargin, forskolin CPA, and ryanodine were each dissolved in dimethyl sulfoxide, to give a final bath concentration <0.1% dimethyl sulfoxide. Ca^{2+}-free Eagle’s minimum essential spinner medium was purchased from Life Technologies, Inc. (Paisley, UK). Papain and collagenase were obtained from Sigma Chemical Co., UK or Worthington Biochemical Corp. (Lakewood, NJ). All other reagents were purchased from Sigma, UK (Poole, UK).
Fig. 1. Ca$^{2+}$ influx, InsP$(1,4,5)$P$_3$, and caffeine each increased $[\text{Ca}^{2+}]_o$. Depolarization from a membrane potential ($V_m$) of $-70$ mV to $0$ mV (C) activated a voltage-dependent Ca$^{2+}$ current (D, and on an expanded time base in E) and elevated $[\text{Ca}^{2+}]_o$ (A). Ins$(1,4,5)$P$_3$ (↑'s in this and following figures) also produced approximately reproducible increases in $[\text{Ca}^{2+}]_o$ (A), as did caffeine (caff.; B).

RESULTS

Depolarization from a membrane potential ($V_m$) of $-70$ mV to $0$ mV (Fig. 1C) activated a voltage-dependent Ca$^{2+}$ current averaging $-160 \pm 18$ pA (Fig. 1D) and a transient increase in $[\text{Ca}^{2+}]_o$, which averaged $1.83 \pm 0.15 \, \Delta F/F_o$ units above baseline ($\triangle F/F_o; n=59; p<0.001$; Fig. 1A). Flash photolysis of caged Ins$(1,4,5)$P$_3$ (Ins$(1,4,5)$P$_3$, upward-pointing arrows) increased $[\text{Ca}^{2+}]_o$, by an average of $2.26 \pm 0.19 \, \Delta F/F_o$ ($n=59; p<0.001$; Fig. 1A). Caffeine (Fig. 1B) elevated $[\text{Ca}^{2+}]_o$ by $2.05 \pm 0.18 \, \Delta F/F_o$ ($n=59; p<0.001$) through activation of RyR. Ins$(1,4,5)$P$_3$ and caffeine each evoked reproducible increases in $[\text{Ca}^{2+}]_o$, when applied at $-50$-s intervals.

Are All InsP$(1,4,5)$P$_3$ Receptors Present on the Ca$^{2+}$ Store Containing RyR?—To test this, the response to Ins$(1,4,5)$P$_3$, following depletion of the ryanodine-sensitive store by caffeine, was examined. At $-70$ mV, Ins$(1,4,5)$P$_3$ evoked approximately reproducible increases in $[\text{Ca}^{2+}]_o$, $(3.28 \pm 0.35 \, \Delta F/F_o; n=5$; Fig. 2A and B) as did caffeine (Fig. 2C), $3.12 \pm 0.3 \, \Delta F/F_o, n=5$; Fig. 2A and B). Caffeine-evoked Ca$^{2+}$ transients were inhibited to $6 \pm 3\%$ of controls by ryanodine (50 μM). $0.14 \pm 0.08 \, \Delta F/F_o; n=5; p<0.001$). Significantly, after this inhibition of the caffeine-evoked Ca$^{2+}$ transient, the Ins$(1,4,5)$P$_3$-evoked Ca$^{2+}$ transient was reduced to $7 \pm 2\%$ of control values ($0.19 \pm 0.04 \, \Delta F/F_o; n=5; p<0.001$; Fig. 2). These results are compatible with the view that Ins$(1,4,5)$P$_3$-R and RyR exist on a common SR Ca$^{2+}$ store.

Does CICR from the RyR Contribute to the InsP$(1,4,5)$P$_3$-evoked Ca$^{2+}$ Transient?—If on the other hand two separate stores exist, i.e. one for Ins$(1,4,5)$P$_3$-R and another for RyR, release of a small amount of Ca$^{2+}$ from the Ins$(1,4,5)$P$_3$-sensitive store could trigger a further, larger release of Ca$^{2+}$ from the separate ryanodine-sensitive store by CICR. If so, depletion of the ryanodine-sensitive store, by caffeine and ryanodine, would reduce the Ins$(1,4,5)$P$_3$-evoked response. If Ca$^{2+}$, released through the Ins$(1,4,5)$P$_3$-R, triggered CICR at the RyR, ryanodine alone would reduce Ins$(1,4,5)$P$_3$-evoked Ca$^{2+}$ transients. This was not observed (Fig. 3). Ins$(1,4,5)$P$_3$ evoked reproducible increases in $[\text{Ca}^{2+}]_o$, of similar magnitude to the presence (50 μM), and absence of ryanodine ($n=5$; Fig. 3, $V_m=-70$ mV). Thus Ca$^{2+}$ released by Ins$(1,4,5)$P$_3$ did not subsequently trigger CICR from the RyR, and this provides further evidence for the existence of a common Ca$^{2+}$ store. In other investigations, reduction, by ryanodine, of the Ca$^{2+}$ transient evoked by Ins$(1,4,5)$P$_3$-generating agents was interpreted as evidence that Ins$(1,4,5)$P$_3$-evoked Ca$^{2+}$ activates CICR at the RyR (11, 28). The plasmalemma agonists used in these experiments to generate Ins$(1,4,5)$P$_3$ could also have activated other second messengers that in turn sensitized the RyR to Ca$^{2+}$ enabling Ins$(1,4,5)$P$_3$-evoked Ca$^{2+}$ release to activate CICR at the RyR. Alternatively, Ca$^{2+}$ release from the SR store may activate further Ca$^{2+}$ release under conditions of “store overload” (31, 32). Such store overload conditions could conceivably arise in some smooth muscle types, facilitating CICR.

To ensure that the absence of an Ins$(1,4,5)$P$_3$-evoked Ca$^{2+}$ transient following depletion of the ryanodine/caffeine-sensitive store (Fig. 2) was due neither to inactivation of the Ins$(1,4,5)$P$_3$-R by caffeine (24, 25) nor to allocation of an inadequate period for store refilling after caffeine, the time course of recovery of the response to Ins$(1,4,5)$P$_3$ after caffeine was examined at $-70$ mV. The magnitude of the Ins$(1,4,5)$P$_3$-evoked transient was reduced to $3 \pm 1\%$ 10 s after caffeine ($n=5$; Fig. 4, A and G). Recovery was time-dependent; 50 s after exposure to caffeine the Ins$(1,4,5)$P$_3$-evoked Ca$^{2+}$ transient
A

B

C

D

E

F

G

Fig. 2. Depletion of the caffeine-sensitive store inhibited the response to InsP(1,4,5)P$_3$. Ins(1,4,5)P$_3$ (A) and caffeine (C) each produced reproducible rises in [Ca$^{2+}$]. (A and B). In the presence of ryanodine (50 μM) the response to caffeine (C) decreased (A and B) as did the response to Ins(1,4,5)P$_3$ (A; A and B). Summary data from five cells are shown in A. *, significant inhibition of the Ins(1,4,5)P$_3$-evoked Ca$^{2+}$ transient; **, significant inhibition of the caffeine-evoked Ca$^{2+}$ transient. 1st, 2nd, etc. refers to the order of responses after treatment in this and other figures.

Fig. 3. Ca$^{2+}$ released through InsP(1,4,5)P$_3$ receptors did not activate RyR. Ins(1,4,5)P$_3$ (A) produced reproducible increases in [Ca$^{2+}$]. (A and B). Ryanodine (50 μM) did not significantly alter the magnitude of Ins(1,4,5)P$_3$-evoked transients, which averaged 1.98 ± 0.37 ΔF/F$_0$, before and 2.02 ± 0.44 ΔF/F$_0$ after ryanodine (n = 5; p > 0.05). A, summarizes data from five cells (mean ± S.E.).

Not All RyR Are Present on the Store, Which Contains InsP(1,4,5)P$_3$R—To determine whether or not all RyR were present on the store that contained Ins(1,4,5)P$_3$, the Ins(1,4,5)P$_3$-sensitive store was depleted by removal of external Ca$^{2+}$ and the ability of caffeine to activate the RyR and evoke a Ca$^{2+}$ transient was examined. Refilling of the Ins(1,4,5)P$_3$-sensitive store is dependent on external Ca$^{2+}$ (33), and removing it reduced the response to Ins(1,4,5)P$_3$ to 5 ± 2% of controls (n = 6; p < 0.05; V_m = −70 mV, Fig. 5). However, after the almost complete loss of the Ins(1,4,5)P$_3$-evoked transient, caffeine evoked a Ca$^{2+}$ transient that averaged 74 ± 25% of control values (n = 6; p < 0.05; Fig. 5). These results are consistent with there being a second separate Ca$^{2+}$ store that contains only RyR.

Refilling of the Ca$^{2+}$ Stores—The above results (Fig. 5) raised the possibility that the degree of dependence of the two stores on external Ca$^{2+}$ for Ca$^{2+}$ release may differ. This was examined following withdrawal of external Ca$^{2+}$ by investigating the refilling of the RyR- and Ins(1,4,5)P$_3$-sensitive stores after either caffeine or Ins(1,4,5)P$_3$. The caffeine-evoked Ca$^{2+}$ transient (via RyR; Fig. 6A) was reduced, on average, to some 87 ± 9% of controls (n = 5; p = 0.5; Fig. 6, A and B). In contrast, the Ins(1,4,5)P$_3$-evoked Ca$^{2+}$ transient (acting through Ins(1,4,5)P$_3$R) was reduced to 6 ± 2% of controls (Fig. 6B; see Fig. 5). These results suggest that, unlike the situation with the Ins(1,4,5)P$_3$-sensitive Ca$^{2+}$ store (Fig. 5), Ca$^{2+}$ release from

had returned to 92 ± 5% of controls (n = 6; Fig. 4, E and G). These results suggest that, at the time intervals used (50–60 s), neither inactivation of the Ins(1,4,5)P$_3$R by caffeine nor an insufficient time period for store refilling accounted for the inhibition of the Ins(1,4,5)P$_3$-evoked Ca$^{2+}$ transient by caffeine and ryanodine (Fig. 2). Collectively, the data (Figs. 2, 3, and 4) suggest that all Ins(1,4,5)P$_3$R exist on a store that also contains RyR.

Fig. 4. Time dependence of the recovery of the InsP(1,4,5)P$_3$-evoked Ca$^{2+}$ transient after caffeine. The magnitude of the Ins(1,4,5)P$_3$-evoked (A) Ca$^{2+}$ transient was virtually abolished 10 s after caffeine (A) from 1.89 ± 0.72 ΔF/F$_0$, to 0.06 ± 0.04 ΔF/F$_0$ (p < 0.05, n = 5). Recovery of the response to Ins(1,4,5)P$_3$ was time-dependent (A–F). The amplitude of the Ins(1,4,5)P$_3$-evoked transient had fully recovered to 3.01 ± 0.9 ΔF/F$_0$, compared with 3.37 ± 1.07 ΔF/F$_0$ before caffeine (p > 0.05; n = 6) 50 s after caffeine (F). The amplitude of both Ins(1,4,5)P$_3$- and caffeine-evoked transients also increased proportionately with time, presumably as the SR Ca$^{2+}$ content increased. G shows summary data from six identical experiments showing the time course of recovery of the Ins(1,4,5)P$_3$-evoked Ca$^{2+}$ transient after caffeine. *, significant inhibition of the Ins(1,4,5)P$_3$-evoked transient (p < 0.05).
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were obtained with forskolin. Removal of external Ca\(^{2+}\) (and addition of 1 mM EGTA) reduced the Ins(1,4,5)P\(_3\)-evoked Ca\(^{2+}\) transient to 5 ± 2% of controls after five UV flashes (from 1.91 ± 0.11 ΔF/ΔF\(_{00}\), to 0.08 ± 0.02 ΔF/ΔF\(_{00}\); n = 6; p < 0.05). Following depletion of the Ins(1,4,5)P\(_3\)-sensitive store, caffeine (caff; C) produced a Ca\(^{2+}\) transient that averaged 74 ± 25% of the control amplitude (1.18 ± 0.4 ΔF/ΔF\(_{00}\), compared with 1.57 ± 0.13 ΔF/ΔF\(_{00}\) prior to depletion of the Ins(1,4,5)P\(_3\)-sensitive store; n = 6; p < 0.05). Data from six cells are summarized in A; *, significant inhibition of the Ins(1,4,5)P\(_3\)-evoked Ca\(^{2+}\) transient; **, significant inhibition of the caffeine-evoked Ca\(^{2+}\) transient.

the RyR by caffeine may be recyled so that refilling is largely independent of external Ca\(^{2+}\).

Effects of Elevation of cAMP on Ins(1,4,5)P\(_3\)-evoked Ca\(^{2+}\) Transients—Caffeine inhibits phosphodiesterase activity and so may elevate the intracellular concentration of cAMP ([cAMP]). The persistence of the store Ca\(^{2+}\) content, in the absence of external Ca\(^{2+}\), as indicated by the maintained amplitude of the caffeine-evoked Ca\(^{2+}\) transient, could have arisen from stimulation of SERCA by an elevated [cAMP], due to caffeine (35) rather than to a difference in the refilling mechanism. To examine this possibility, dependence of Ins(1,4,5)P\(_3\) store refilling on external Ca\(^{2+}\) was examined when [cAMP] had been increased (a) by the phosphodiesterase inhibitor IBMX (500 μM) and (b) by forskolin (1 μM), which stimulates adenylate cyclase thereby raising [cAMP]. In the absence of either drug, Ins(1,4,5)P\(_3\)-evoked Ca\(^{2+}\) transients of approximately reproducible amplitude that averaged 1.89 ± 0.12 ΔF/ΔF\(_{00}\) (n = 6). Following incubation (10 min) with either IBMX or forskolin, Ins(1,4,5)P\(_3\)-evoked transients of approximately reproducible amplitude (2.18 ± 0.67 ΔF/ΔF\(_{00}\); n = 6; Fig. 7, for IBMX), which were not significantly different from controls. Upon removal of external Ca\(^{2+}\), in the continued presence of either IBMX or forskolin, repeated application of Ins(1,4,5)P\(_3\) depleted the Ins(1,4,5)P\(_3\)-sensitive store as evidenced by the decline in the amplitude of the Ca\(^{2+}\) transient. With IBMX, after the fourth Ins(1,4,5)P\(_3\) challenge, the Ca\(^{2+}\) increase averaged 15 ± 3% of the Ins(1,4,5)P\(_3\)-evoked Ca\(^{2+}\) transients observed in IBMX in the presence of external Ca\(^{2+}\) (0.56 ± 0.31 ΔF/ΔF\(_{00}\); p < 0.01; n = 6; Fig. 7). Qualitatively similar results were obtained with forskolin. Removal of external Ca\(^{2+}\) again inhibited the amplitude of the Ins(1,4,5)P\(_3\)-evoked transient significantly to 8 ± 3% of controls (p < 0.05 by Mann-Whitney test; data not shown; V\(_{m}\) = −70 mV). In these same cells only 3 ± 3% of the Ins(1,4,5)P\(_3\)-evoked transient remained in the presence of forskolin (1 μM) following the removal of external Ca\(^{2+}\) (n = 3; p < 0.05 by Mann-Whitney test). Together the results with IBMX and forskolin indicated that elevation of [cAMP], is unlikely to offset the effect of external Ca\(^{2+}\) withdrawal on store content.

Effects of SR Ca\(^{2+}\) Pump Inhibitors—Ca\(^{2+}\) stores have been differentiated on the basis of their sensitivity to the SERCA inhibitors cyclopiazonic acid (CPA) and thapsigargin (5, 36, 37). The ability of CPA and thapsigargin to each inhibit Ins(1,4,5)P\(_3\) and caffeine-evoked Ca\(^{2+}\) transients was therefore examined. Cells were once again held at a membrane potential of −70 mV. Ins(1,4,5)P\(_3\) and caffeine (Fig. 6, C and F) each produced reproducible increases in Ca\(^{2+}\) at ~50 s intervals (Fig. 6, B and E). Thapsigargin (500 nM) increased resting [Ca\(^{2+}\)]\(_o\) from 1.07 ± 0.05 ΔF/ΔF\(_{00}\) to 1.73 ± 0.12 ΔF/ΔF\(_{00}\) after 5 min (n = 10; p < 0.001; Fig. 8B), and inhibited the responses to both Ins(1,4,5)P\(_3\) and caffeine (Fig. 8 A and B). Ins(1,4,5)P\(_3\)-evoked Ca\(^{2+}\) transients were reduced, from an average of 4.19 ± 0.40 ΔF/ΔF\(_{00}\) to 0.23 ± 0.06 ΔF/ΔF\(_{00}\), in the presence of thapsigargin (n = 10; p < 0.001; Fig. 8A). The caffeine-evoked Ca\(^{2+}\) transient was also reduced from 3.81 ± 1.38 ΔF/ΔF\(_{00}\) to 0.04 ± 0.06 ΔF/ΔF\(_{00}\) in the presence of thapsigargin (n = 10; p < 0.001; Fig. 8A). CPA (10 μM) also increased resting [Ca\(^{2+}\)]\(_o\) from 1.18 ± 0.09 ΔF/ΔF\(_{00}\) immediately prior to CPA to 1.46 ± 0.11 ΔF/ΔF\(_{00}\) after 5 min in the drug (n = 16; p < 0.001; Fig. 8E). CPA inhibited both the Ins(1,4,5)P\(_3\)-evoked and caffeine-evoked Ca\(^{2+}\) transients (Fig. 8 D and E). On average, the response to Ins(1,4,5)P\(_3\) was reduced from 1.70 ± 0.32 ΔF/ΔF\(_{00}\) to 0.09 ± 0.04 ΔF/ΔF\(_{00}\) in CPA (n = 16; p < 0.001; Fig. 8D) and that to caffeine from 1.65 ± 0.34 ΔF/ΔF\(_{00}\) to 0.07 ± 0.06 ΔF/ΔF\(_{00}\) (n = 16; p < 0.001; Fig. 8D). Thus, refilling of both the Ins(1,4,5)P\(_3\)-sensitive and caffeine-sensitive stores is prevented by each of the SERCA inhibitors thapsigargin and CPA; use of SERCA inhibitors is unlikely to be of value in differentiating between Ca\(^{2+}\) stores in these cells.

**DISCUSSION**

Ca\(^{2+}\) release from and uptake into internal SR Ca\(^{2+}\) stores plays a central role in the activity of most cells, including that of excitation-contraction coupling in smooth muscle. Although it is conceded that release occurs via Ins(1,4,5)P\(_3\),R and RyR, the interrelationship between these two receptors in their access to stored Ca\(^{2+}\) is unclear. A diversity of store and receptor arrangements has been proposed. This study has demonstrated the presence of two Ca\(^{2+}\) stores in colonic smooth muscle, one expressing both Ins(1,4,5)P\(_3\)R and RyR, the other only RyR. Support for this view comes from two key observations in which the stores accessed by either Ins(1,4,5)P\(_3\)R or RyR were depleted of Ca\(^{2+}\) . First, the store accessed by RyR was depleted of Ca\(^{2+}\) (by repetitive activation with caffeine in the presence of ryanodine to maintain the RyR in the open state), and under these circumstances the response to Ins(1,4,5)P\(_3\) was virtually abolished. Second, when the store accessed by Ins(1,4,5)P\(_3\)R was depleted, by withdrawal of external Ca\(^{2+}\), and the Ins(1,4,5)P\(_3\) response was effectively abolished, significantly, the Ca\(^{2+}\) response to RyR activation by caffeine persisted. The observation that the Ins(1,4,5)P\(_3\)-evoked Ca\(^{2+}\) response was almost abolished after caffeine (in the presence of ryanodine) could not be attributed to inhibition of an amplification of the Ins(1,4,5)P\(_3\)-evoked response by CICR, because Ins(1,4,5)P\(_3\)-evoked Ca\(^{2+}\) release did not activate this mechanism. Nor could the observation be explained by inactivation of the Ins(1,4,5)P\(_3\)-R by caffeine because the Ins(1,4,5)P\(_3\)-evoked transient had fully recovered at the time intervals between application of caffeine and Ins(1,4,5)P\(_3\) used in the present...
study. Finally, the results of the present study demonstrated differences in the refilling mechanisms of the two stores. The store expressing both Ins(1,4,5)P³R and RyR was dependent on external Ca²⁺ for replenishment whereas the store with only RyR was not.

The precise number and arrangement of Ca²⁺ stores, among...
different cell types, is a matter of debate. In cerebellar Purkinje neurons, as in the present study, ryanodine inhibited the Ins(1,4,5)P3-evoked Ca2+ transient, in keeping with the view that both Ins(1,4,5)P3R and RyR were present on a common store. However, whether or not an additional separate store with only RyR involved was not determined (38). One store with both Ins(1,4,5)P3R and RyR and an additional separate store, with only RyR similar to the present view, have also been proposed in vascular smooth muscle (21). Other studies had initially raised the possibility of there being two stores but with different receptor arrangements from those presently proposed. For example, from experiments in smooth muscle, of the two Ca2+ stores proposed, one contained both RyR and Ins(1,4,5)P3R, the other only Ins(1,4,5)P3R (19, 20). Alternatively, separate stores have been proposed for both RyR and Ins(1,4,5)P3 receptors (e.g. 39–41). A commonly employed method with which to study the receptors on the Ca2+ stores has been to inhibit store Ca2+ pumps by thapsigargin or cyclopiazonic acid (CPA) and to observe the responses to activation of Ins(1,4,5)P3R and RyR. In various cell types, thapsigargin and CPA each abolished Ca2+ release in response to activation of either RyR or Ins(1,4,5)P3R but not to activation of both. This supported the idea of there being more than one store (36, 37, 42–44). Studies in smooth muscle and astrocytes suggested that, despite an apparently uniform Ca2+ content throughout most of the store, CPA and the RyR activator caffeine released Ca2+ from seemingly separate compartments (5, 22), i.e. there were in effect two stores. However, the relationship between SR Ca2+ pumps on the one hand and the Ins(1,4,5)P3R and RyR on the other could not be presently differentiated by the use of Ca2+ pump inhibitors. SR Ca2+ pumps presumably on both the common Ins(1,4,5)P3/RyR store and the RyR-only store were each inhibited by thapsigargin and CPA (see also 45, 46). Differences in the sensitivity of the Ca2+ pumps on the internal SR Ca2+ store to the inhibitors (47, 48), which may also vary among different tissues, were presumably responsible for these observations.

Differences in SR Luminal Ca2+ Regulation of the Receptors Does Not Account for the Differences in Response to Ins(1,4,5)P3 and Caffeine—On the basis of the two-store system proposed, the observation that a substantial caffeine-evoked Ca2+ transient persisted, after depletion of the Ins(1,4,5)P3-sensitive store, could be explained if the opening of the Ins(1,4,5)P3/RyR store and the RyR-only store were each inhibited by thapsigargin and CPA (see also 45, 46). Differences in the sensitivity of the Ca2+ pumps on the internal SR Ca2+ store to the inhibitors (47, 48), which may also vary among different tissues, were presumably responsible for these observations. In the store containing both RyR and Ins(1,4,5)P3R, the other only Ins(1,4,5)P3R (19, 20). Alternatively, separate stores have been proposed for both RyR and Ins(1,4,5)P3 receptors (e.g. 39–41). A commonly employed method with which to study the receptors on the Ca2+ stores has been to inhibit store Ca2+ pumps by thapsigargin or cyclopiazonic acid (CPA) and to observe the responses to activation of Ins(1,4,5)P3R and RyR. In various cell types, thapsigargin and CPA each abolished Ca2+ release in response to activation of either RyR or Ins(1,4,5)P3R but not to activation of both. This supported the idea of there being more than one store (36, 37, 42–44). Studies in smooth muscle and astrocytes suggested that, despite an apparently uniform Ca2+ content throughout most of the store, CPA and the RyR activator caffeine released Ca2+ from seemingly separate compartments (5, 22), i.e. there were in effect two stores. However, the relationship between SR Ca2+ pumps on the one hand and the Ins(1,4,5)P3R and RyR on the other could not be presently differentiated by the use of Ca2+ pump inhibitors. SR Ca2+ pumps presumably on both the common Ins(1,4,5)P3/RyR store and the RyR-only store were each inhibited by thapsigargin and CPA (see also 45, 46). Differences in the sensitivity of the Ca2+ pumps on the internal SR Ca2+ store to the inhibitors (47, 48), which may also vary among different tissues, were presumably responsible for these observations.

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Mechanisms of Store Refilling—Depletion of Ins(1,4,5)P3-sensitive stores in all cell types so far examined activates a store-operated Ca2+ entry pathway, which is necessary for its replenishment (reviewed in Refs. 56–58) as is depletion of the ryanodine-sensitive store, although not in all cell types (59–63). In the present study, replenishment of the store containing RyR alone did not require external Ca2+, suggesting that plasmalemmal store-operated Ca2+ entry is unnecessary for the maintenance of the Ca2+ content of this store. In the store containing both RyR and Ins(1,4,5)P3R, on the other hand, external Ca2+ entry, presumably via store-operated channels, is essential for store refilling in colonic myocytes (33). Perhaps differences in store location within the cell determines the external Ca2+ dependence of the refilling mechanisms. The store containing both RyR and Ins(1,4,5)P3R may be positioned closer to the plasmalemma than that containing only RyR and may be functionally more closely linked to Ca2+ entry via store-operated channels (63). Different isoforms of SERCA, with different Ca2+ binding affinities, e.g. Kd, 0.31 ± 0.02 μM and 0.17 ± 0.01 μM Ca2+ for SERCA2a and SERCA2b, respectively (Ref. 42 for review; see also Refs. 65–67), may be asso-
ciated with the separate stores and could explain the ability of the SR to refill in the presence of different [Ca2+]c. Ca2+ uptake into stores by SERCA2b may also be modulated by calmodulin (68), phospha-loban (64, 67), and calrectulin and calnexin (26) so that differential modulation of Ca2+ pumps may also help to explain differences in store refilling in the absence of external Ca2+.

The present study emphasizes the complexity of the organi-
zation of the SR Ca2+ stores. It proposes the existence of two in the control of Ca2+ release; one containing both RyR and Ins(1,4,5)P3R and a second only RyR. The results also highlight the possibility of structural components within the SR, each with their unique content of receptors/channels permitting local control of the SR Ca2+ signal in response to receptor modulation.

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