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$^{2+}$]$_{cyt}$). 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 clonial 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$_3$R-sensitive store, by the removal of external Ca$^{2+}$, on the other hand, caused only a small decrease (~28%) 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$_3$R-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$_3$R-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 refilled from [Ca$^{2+}$]$_{lumen}$, the second expressing both Ins(1,4,5)P$_3$R and RyR and dependent upon external Ca$^{2+}$ for refilling.

Release of Ca$^{2+}$ from the sarcoplasmic reticulum (SR)$^2$ store, a mechanism that regulates smooth muscle contractile activity, involves the participation of two receptor/channel complexes, the ryanodine receptor (RyR) and the inositol 1,4,5-trisphosphate receptor (Ins(1,4,5)P$_3$R). Release from this store regulates the bulk average [Ca$^{2+}$]$_{cyt}$, both directly (1) and indirectly either via modulation of the plasmalemmal membrane potential (2) or by activation of store-operated Ca$^{2+}$ entry (3). The magnitude, time course, and frequency of the SR Ca$^{2+}$ signal depend on the functional interaction, localization, and arrangement of the Ins(1,4,5)P$_3$R and RyR on the SR store(s).

Although, morphologically, the SR appears as an interconnected network of tubules (4, 5), it may adopt different configurations within the cell and components may detach and realign thereby influencing the pattern and distribution of the RyR and Ins(1,4,5)P$_3$R (6, 7). In Purkinje neurons, for example, Ins(1,4,5)P$_3$R-expressing regions may detach from other internal store elements (8, 9). Indeed, different Ca$^{2+}$ concentrations have been found within the lumen of the SR (10) suggesting that discontinuities may exist within the structures surrounding the lumen itself. This provides a morphological basis for the existence of various arrangements of Ca$^{2+}$ stores.

The SR Ca$^{2+}$ stores in smooth muscle are classified on the basis of the arrangement of Ins(1,4,5)P$_3$R and RyR; yet conflicting evidence exists regarding their number. A single store, containing both RyR and Ins(1,4,5)P$_3$R, has been proposed, based on the observation that caffeine (which activates RyR) prevented Ins(1,4,5)P$_3$-mediated Ca$^{2+}$ release (e.g. 11–16). Two separate Ca$^{2+}$ stores have also been proposed, one that expresses only RyR, the other only Ins(1,4,5)P$_3$R. In support of this latter view, depletion of the RyR-sensitive store failed to abolish agonist-evoked Ins(1,4,5)P$_3$-mediated Ca$^{2+}$ release and vice versa (17). More elaborate arrangements of SR Ca$^{2+}$ stores have also been proposed. In some smooth muscles (e.g. taenia coli, pulmonary artery, myometrium) one store may express both RyR and Ins(1,4,5)P$_3$R, whereas a second, in the same cell, may express Ins(1,4,5)P$_3$R alone (18–20). Conversely, one store expressing both RyR and Ins(1,4,5)P$_3$R and a second separate store RyR alone have also been suggested (21).

Further support for the existence of two separate stores has come from studies on the response of each of the receptors to inhibitors of the SR Ca$^{2+}$ pump, thapsigargin and cyclopiazonic acid (CPA), each of which depletes the stores of Ca$^{2+}$. Differences in the sensitivity to the pump inhibitors of the Ca$^{2+}$ release evoked by either caffeine or Ins(1,4,5)P$_3$ have been interpreted as evidence for the existence of separate stores for each receptor (5, 16, 22). For example, in arterial myocytes the ryanodine/caffeine-sensitive store was not sensitive to either thapsigargin or CPA, whereas the Ins(1,4,5)P$_3$-sensitive Ca$^{2+}$ store was depleted by each (16, 22). The situation has been complicated further by the proposed existence in murine blad-
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\(^{2+}\)]\(_c\), 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 multicellular 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\(_2\) 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\(^{2+}\)]\(_c\).
Depolarization from a membrane potential \( V_m \) of \(-70 \text{ mV} \) to 0 mV (Fig. 1C) activated a voltage-dependent \( Ca^{2+} \) current (D, and on an expanded time base in E) and elevated \([Ca^{2+}]_o\) (A). Ins(1,4,5)P_3 (†’s in this and following figures) also produced approximately reproducible increases in \([Ca^{2+}]_i\) (A), as did caffeine (caff.; B).

RESULTS

Depolarization from a membrane potential \( V_m \) of \(-70 \text{ mV} \) to 0 mV (Fig. 1C) activated a voltage-dependent \( Ca^{2+} \) current averaging \(-160 \pm 18 \text{ pA} \) (Fig. 1D) and a transient increase in \([Ca^{2+}]_o\), which averaged \(-1.83 \pm 0.15 \text{ pA} \) units above baseline (\(\Delta [Ca^{2+}]_o; n = 59; p < 0.001\); Fig. 1A). Flash photolysis of caged Ins(1,4,5)P_3 (Ins(1,4,5)P_3, upward-pointing arrows) increased \([Ca^{2+}]_i\), by an average of \(2.26 \pm 0.19 \text{ pA} (n = 59; p < 0.001; \) Fig. 1A). Caffeine (Fig. 1B) elevated \([Ca^{2+}]_i\), by 2.05 \(\pm 0.18 \text{ pA} (n = 59; p < 0.001) \) through activation of RyR. Ins(1,4,5)P_3 and caffeine each evoked reproducible increases in \([Ca^{2+}]_i\), when applied at \(-50-\text{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 \(-70 \text{ mV}, \) Ins(1,4,5)P_3 evoked approximately reproducible increases in \([Ca^{2+}]_i\), \((3.28 \pm 0.35 \text{ pA}; n = 5; \) Fig. 2, A and B) as did caffeine (Fig. 2C, \(3.12 \pm 0.2 \text{ pA}; n = 5; \) Fig. 2, A and B). Caffeine-evoked \( Ca^{2+} \) transients were inhibited to 6 \(\pm 3\% \) of controls by ryanodine (50 \text{ \mu M}; \(0.14 \pm 0.08 \text{ pA}; n = 5; p < 0.001\)). 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 \(\pm 0.04 \text{ pA}; n = 5; p < 0.001; \) Fig. 2). These results are compatible with the view that Ins(1,4,5)P_3 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 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_3R, 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 \([Ca^{2+}]_i\), of similar magnitude in the presence (50 \text{ \mu M}) and absence of ryanodine (n = 5; Fig. 3, \(V_m = -70 \text{ mV} \)). 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 inad-
A cells are shown in ryanodine (50 \mu M) the response to caffeine (C) decreased (A and B) as did the response to Ins(1,4,5)P3 (A and B). Summary data from five cells are shown in A. *, significant inhibition of the Ins(1,4,5)P3-evoked Ca2+ transient; **, significant inhibition of the caffeine-evoked Ca2+ transient. 1st, 2nd, etc. refers to the order of responses after treatment in this and other figures.

The magnitude of the Ins(1,4,5)P3-evoked ( \( \Delta F/F_o \) ) Ca2+ transient was virtually abolished 10 s after caffeine (A) from 1.89 ± 0.72 \( \Delta F/F_o \) , to 0.06 ± 0.04 \( \Delta F/F_o \) (p < 0.05, n = 5). Recovery of the response to Ins(1,4,5)P3 was time-dependent (A–F). The amplitude of the Ins(1,4,5)P3-evoked transient had fully recovered to 3.01 ± 0.9 \( \Delta F/F_o \), compared with 3.37 ± 1.07 \( \Delta F/F_o \), before caffeine (p > 0.05; n = 6) 50 s after caffeine (F). The amplitude of both Ins(1,4,5)P3- and caffeine-evoked transients also increased proportionately with time, presumably as the SR Ca2+ content increased. G shows summary data from six identical experiments showing the time course of recovery of the Ins(1,4,5)P3-evoked Ca2+ transient after caffeine. +, significant inhibition of the Ins(1,4,5)P3-evoked transient (p < 0.05).

**Not All RyR Are Present on the Store, Which Contains InsP(1,4,5)P3R**—To determine whether or not all RyR were present on the store that contained Ins(1,4,5)P3R, the Ins(1,4,5)P3-sensitive store was depleted by removal of external Ca2+ and the ability of caffeine to activate the RyR and evoke a Ca2+ transient was examined. Refilling of the Ins(1,4,5)P3-sensitive store is dependent on external Ca2+ (33), and removing it reduced the response to Ins(1,4,5)P3 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)P3-evoked transient, caffeine evoked a Ca2+ 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 Ca2+ store that contains only RyR.

**Refilling of the Ca2+ Stores**—The above results (Fig. 5) raised the possibility that the degree of dependence of the two stores on external Ca2+ for Ca2+ release may differ. This was examined following withdrawal of external Ca2+ by investigating the refilling of the RyR- and Ins(1,4,5)P3R-sensitive stores after either caffeine or Ins(1,4,5)P3. The caffeine-evoked Ca2+ 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)P3-evoked Ca2+ transient (acting through Ins(1,4,5)P3R) 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)P3-sensitive Ca2+ store (Fig. 5), Ca2+ release from...
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were obtained with forskolin. Removal of external Ca\(^{2+}\); *, significant inhibition of the Ins(1,4,5)P\(_3\)-evoked Ca\(^{2+}\) transient; **, significant inhibition of the Ins(1,4,5)P\(_3\)-evoked Ca\(^{2+}\) transient after the fourth Ins(1,4,5)P\(_3\) challenge, the Ca\(^{2+}\) transient had fully recovered at the time intervals between application of caffeine and Ins(1,4,5)P\(_3\) used in the present test; data not shown; \(V_m = -70\) mV. In these same cells only 3 ± 2% of the Ins(1,4,5)P\(_3\)-evoked transient remained in the presence of forskolin (1 \(\mu\)M) 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 about -70 mV. Ins(1,4,5)P\(_3\) and caffeine (Fig. 8, C and F) each produced reproducible increases in [Ca\(^{2+}\)], at ~50-s intervals (Fig. 8, B and E). Thapsigargin (500 nM) increased resting [Ca\(^{2+}\)] from 1.07 ± 0.05 F/F\(_o\) to 1.73 ± 0.12 F/F\(_o\) 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. 8A and B). Ins(1,4,5)P\(_3\)-evoked Ca\(^{2+}\) transients were reduced, from an average of 4.19 ± 0.40 ΔF/F\(_o\) in control to 0.23 ± 0.06 ΔF/F\(_o\) 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/F\(_o\) in control to -0.04 ± 0.06 ΔF/F\(_o\) in the presence of thapsigargin (n = 10; p < 0.001; Fig. 8A). CPA (10 μM) also increased resting [Ca\(^{2+}\)], from 1.18 ± 0.09 F/F\(_o\) immediately prior to CPA to 1.46 ± 0.11 F/F\(_o\) 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/F\(_o\) to 0.09 ± 0.04 ΔF/F\(_o\) in CPA (n = 16; p < 0.001; Fig. 8D) and that to caffeine from 1.65 ± 0.34 ΔF/F\(_o\) to 0.07 ± 0.06 ΔF/F\(_o\) (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.

Fig. 5. Depletion of the InsP(1,4,5)P\(_3\)-sensitive store reduced but did not abolish the response to caffeine. Ins(1,4,5)P\(_3\) (\(\uparrow\)) and caffeine (C) each evoked approximately reproducible increases in [Ca\(^{2+}\)]. (A and B; \(V_m = -70\) mV). 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/F\(_o\) to 0.08 ± 0.02 ΔF/F\(_o\); 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/F\(_o\) compared with 1.57 ± 0.13 ΔF/F\(_o\) 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.

Effects of Elevation of cAMP on InsP(1,4,5)P\(_3\)-evoked Ca\(^{2+}\) Transients—Caffeine inhibits phosphodiesterase activity and so may elevate the intracellular concentration of cAMP ([cAMP]c). 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]c, 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]c had been increased (a) by the phosphodiesterase inhibitor IBMX (500 μM) and (b) by forskolin (1 μM), which stimulates adenylate cyclase thereby raising [cAMP]c. 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/F\(_o\) (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/F\(_o\); 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/F\(_o\); 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).
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)P$_3$-evoked Ca$^{2+}$ transient, in keeping with the view that both Ins(1,4,5)P$_3$R 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)P$_3$R 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 Ca$^{2+}$ stores proposed, one contained both RyR and Ins(1,4,5)P$_3$R, the other only Ins(1,4,5)P$_3$R (19, 20). Alternatively, separate stores have been proposed for both RyR and Ins(1,4,5)P$_3$ receptors (e.g. 39–41). A commonly employed method with which to study the receptors on the Ca$^{2+}$ stores has been to inhibit store Ca$^{2+}$ pumps by thapsigargin or cyclopiazonic acid (CPA) and to observe the responses to activation of Ins(1,4,5)P$_3$R and RyR. In various cell types, thapsigargin and CPA each abolished Ca$^{2+}$ release in response to activation of either RyR or Ins(1,4,5)P$_3$R 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 Ca$^{2+}$ content throughout most of the store, CPA and the RyR activator caffeine released Ca$^{2+}$ from seemingly separate compartments (5, 22), i.e. there were in effect two stores. However, the relationship between SR Ca$^{2+}$ pumps on the one hand and the Ins(1,4,5)P$_3$R and RyR on the other could not be presently differentiated by the use of Ca$^{2+}$ pump inhibitors. SR Ca$^{2+}$ pumps presumably on both the common Ins(1,4,5)P$_3$/RyR store and the RyR-only store were each inhibited by thapsigargin and CPA (see also 45, 46). Differences in the sensitivity of the Ca$^{2+}$ pumps on the internal SR Ca$^{2+}$ store to the inhibitors (47, 48), which may also vary among different tissues, were presumably responsible for these observations.

**Differences in SR Luminal Ca$^{2+}$ Regulation of the Receptors Does Not Account for the Differences in Response to Ins(1,4,5)P$_3$ and Caffeine**—On the basis of the two-store system proposed, the observation that a substantial caffeine-evoked Ca$^{2+}$ transient persisted, after depletion of the Ins(1,4,5)P$_3$-sensitive store, could be explained if the opening of the Ins(1,4,5)P$_3$/RyR store and the RyR-only store were each inhibited by thapsigargin and CPA (see also 45, 46). Differences in the sensitivity of the Ca$^{2+}$ pumps on the internal SR Ca$^{2+}$ store to the inhibitors (47, 48), which may also vary among different tissues, were presumably responsible for these observations.

**Mechanisms of Store Refilling**—Depletion of Ins(1,4,5)P$_3$-sensitive stores in all cell types so far examined activates a store-operated Ca$^{2+}$ 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 Ca$^{2+}$, suggesting that plasmalemmal store-operated Ca$^{2+}$ entry is unnecessary for the maintenance of the Ca$^{2+}$ content of this store. In the store containing both RyR and Ins(1,4,5)P$_3$R on the other hand, external Ca$^{2+}$ 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 Ca$^{2+}$ dependence of the refilling mechanisms. The store containing both RyR and Ins(1,4,5)P$_3$R may be positioned closer to the plasmalemma than that containing only RyR and may be functionally more closely linked to Ca$^{2+}$ entry via store-operated channels (63). Different isoforms of SERCA, with different Ca$^{2+}$ binding affinities, e.g. $K_a$ = 0.31 ± 0.02 μM and 0.17 ± 0.01 μM Ca$^{2+}$ 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 [Ca$^{2+}$]. Ca$^{2+}$ uptake into stores by SERCA2b may also be modulated by calmodulin (68), phospholamban (64, 67), and calreticulin and calnexin (26) so that differential modulation of Ca$^{2+}$ pumps may also help to explain differences in store refilling in the absence of external Ca$^{2+}$.

The present study emphasizes the complexity of the organization of the SR Ca$^{2+}$ stores. It proposes the existence of two in the control of Ca$^{2+}$ release; one containing both RyR and Ins(1,4,5)P$_3$R 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 Ca$^{2+}$ signal in response to receptor modulation.

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**REFERENCES**

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