The ability to change the cytosolic free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_{c}\)) is an inherent characteristic of eukaryotic cells. The amplitude, duration, frequency, and location of each of these [Ca\(^{2+}\)]\(_{c}\) changes are important determinants of the Ca\(^{2+}\) signal; they combine to target specific cellular activities such as gene expression and metabolism and so control the nature of the overall cellular response. The cell's ability to modulate each of these aspects of Ca\(^{2+}\) change provides the flexibility required to enable a single messenger to generate and manage many different and often opposing cellular functions.

Two main sources of [Ca\(^{2+}\)]\(_{c}\) are recognized: the extracellular fluid and the intracellular stores of the sarcoplasmic reticulum (SR). One of the main Ca\(^{2+}\) entry pathways from the extracellular fluid is the voltage-dependent Ca\(^{2+}\) channel in the sarcolemma. Membrane depolarization increases the activity of these channels to enhance Ca\(^{2+}\) entry and raise [Ca\(^{2+}\)]\(_{c}\). The other main [Ca\(^{2+}\)]\(_{c}\) source is the internal SR store from which release proceeds via two main receptor-controlled channels: the inositol 1,4,5-trisphosphate (IP\(_3\)) receptor (IP\(_3\)R) and the ryanodine receptor (RyR). The SR may liberate Ca\(^{2+}\) in response to activation of IP\(_3\)R as a result of agonist activity at the sarcolemma that increases IP\(_3\) production. Ca\(^{2+}\) influx from outside may activate RyR in the process of Ca\(^{2+}\)-induced Ca\(^{2+}\) release (CICR). The SR may also release Ca\(^{2+}\), via RyR, when the store's Ca\(^{2+}\) content exceeds normal physiological values, i.e., in "store overload." Indeed, store overload may facilitate the occurrence of CICR in cells that do not normally display this feature.

In our studies, depolarization activates voltage-dependent Ca\(^{2+}\) channels to increase [Ca\(^{2+}\)]\(_{c}\) uniformly throughout the bulk of the cytoplasm (Fig. 1A). The increase can be explained solely by Ca\(^{2+}\) influx and does not involve the SR or CICR (3). Thus depolarization does not increase [Ca\(^{2+}\)]\(_{c}\) in the presence of voltage-dependent Ca\(^{2+}\) channel blockers, in the absence of extracellular Ca\(^{2+}\), or when the membrane potential is held at the equilibrium potential for Ca\(^{2+}\). Each of these findings demonstrates that, in response to sarcolemma depolarization, Ca\(^{2+}\) influx is essential for a rise in [Ca\(^{2+}\)]\(_{c}\), and that channel activity or membrane potential changes themselves failed to alter [Ca\(^{2+}\)]\(_{c}\) in the absence of influx. Moreover, Ca\(^{2+}\) influx produced by depolarization of the sarcolemma is quantitatively adequate to account for the entire [Ca\(^{2+}\)]\(_{c}\) increase, without SR involvement (3). At least 100 times more Ca\(^{2+}\) enters the cell than appears as free Ca\(^{2+}\) in the cytosol (3). Not surprisingly, therefore, depletion of the SR Ca\(^{2+}\) stores using the Ca\(^{2+}\) pump inhibitors thapsigargin or cyclopiazonic acid or by ryanodine and caffeine to open RyR on the SR (Fig. 1B) does not reduce depolarization-evoked [Ca\(^{2+}\)]\(_{c}\) increases (3). These results suggest that the SR does not contribute to the rise in [Ca\(^{2+}\)]\(_{c}\) evoked by Ca\(^{2+}\) influx via CICR, although controversy persists as to the precise contribution of CICR in smooth muscle. Significantly, in equine tracheal myocytes, Ca\(^{2+}\) influx may activate, albeit after a considerable delay, Ca\(^{2+}\) release from the internal SR store in a process referred to as "loosely
Figure 1, continued. Ryanodine increased the measured [Ca²⁺]c value for a given calculated Ca²⁺ value compared with that in its absence (control; n = 15); i.e., ryanodine decreased the apparent Ca²⁺ buffer capacity of the cell. Reproduced from Cell Calcium (2) with permission. C: thapsigargin depleted the SR Ca²⁺ content but did not increase the depolarization-evoked rise in [Ca²⁺]c. Depolarization (~70 mV to 0 mV, c) triggered an inward Ca²⁺ current (d) and a Ca²⁺ transient (a). Caffeine (10 mM, b) and IP₃ (arrow) each produced Ca²⁺ transients (a). Thapsigargin (500 nM) abolished both (a; note the residual flash artifact indicated by the arrow), indicating that the SR Ca²⁺ content had been significantly reduced. Thapsigargin (500 nM) reduced peak amplitude of the Ca²⁺ current (d and e), and as a consequence the magnitude of the depolarization-evoked Ca²⁺ transient (a). However, in contrast to the effects of ryanodine, the relationship between the amount of Ca²⁺ entering the cell by depolarization i.e., the calculated [Ca²⁺]c (see Ref. 3 for method of calculation) and the measured [Ca²⁺]c was not significantly altered by thapsigargin (n = 8; P > 0.05); i.e., the apparent Ca²⁺ buffer capacity of the cell was not decreased by thapsigargin. Reproduced from Cell Calcium (2) with permission. D: passive Ca²⁺ trap in smooth muscle. Ca²⁺ entering the cell is retained in a buffer region from which the ion only slowly escapes by negotiating a restricted space between the SR and sarcolemma (a). The SR may also restrict access of bulk [Ca²⁺]c to the sarcolemma (b). When RyR are locked open, the SR is rendered “leaky.” Ca²⁺ is not retained in the trap but may move more freely through the SR structure to exit into the bulk of the cytoplasm; i.e., more of the Ca²⁺ entering the cell during transient depolarizations appears as free Ca²⁺ in the bulk cytosol. With a leaky SR (c), when Ca²⁺ influx is terminated and removal mechanisms dominate to control [Ca²⁺]c, the Ca²⁺ gradients within the cell are inverted and then the process is reversed; Ca²⁺ must reach the sarcolemma to be expelled from the cell. SR Ca²⁺ pump (SERCA) activity and a leaky SR may increase the rate at which the ion reaches the sarcolemma. SLCA, sarcolemma Ca²⁺ pump. Reproduced from Cell Calcium (2) with permission.
coupled CICR" (10). The delayed Ca\(^{2+}\) release from the SR was attributed to the time required for subsarcolemmal [Ca\(^{2+}\)] to build up in the gap between the voltage-dependent Ca\(^{2+}\) channels in the sarcolemma and the RyR in the SR to the value necessary to activate CICR. However, the delay between Ca\(^{2+}\) influx and Ca\(^{2+}\) release from the SR may also reflect the time required for the SR store to accumulate and subsequently discharge Ca\(^{2+}\) via RyR when store overload conditions had developed. In those cells in which "loosely coupled CICR" has been described, experiments are required to distinguish between store overload-evoked Ca\(^{2+}\) release and the proposed loosely coupled CICR.

Rather than contributing to an increase in [Ca\(^{2+}\)]\(_i\), the SR may in fact limit the rise that occurs after influx. SR pump activity reportedly contributes to this limiting effect of the SR in the "superficial buffer barrier" hypothesis (see Ref. 2 for references). Our own observations, however, suggest that pump activity does not substantially reduce the bulk average [Ca\(^{2+}\)]\(_i\) rise that occurs during depolarization (2). Although unable to modify the rise in [Ca\(^{2+}\)]\(_i\) occurring during influx, the SR accumulates Ca\(^{2+}\) and contributes to the decline in [Ca\(^{2+}\)]\(_i\) when influx ends by pump activity throughout the SR structure (3).

The increased SR Ca\(^{2+}\) load after sarcolemma depolarization (as a result of its accumulation of the ion) is exported slowly to the outside of the cell by Na\(^+/\)Ca\(^{2+}\) exchanger activity at the sarcolemma (as a result of its accumulation of the ion) is exported slowly to the outside of the cell by Na\(^+/\)Ca\(^{2+}\) exchanger activity at the sarcolemma to restore the steady-state SR Ca\(^{2+}\) content (3).

Although SR Ca\(^{2+}\) pump activity does not limit the rise in [Ca\(^{2+}\)]\(_i\), the SR itself nonetheless restricts the increase in bulk [Ca\(^{2+}\)]\(_i\) that follows influx by forming a passive Ca\(^{2+}\) trap.

Inducing leakage of the SR store, using ryanodine together with caffeine (and fully depleting the SR of Ca\(^{2+}\)), significantly increases the [Ca\(^{2+}\)]\(_i\) rise that occurs after depolarization and Ca\(^{2+}\) influx (Fig. 1B). Yet fully depleting the SR by using either thapsigargin (Fig. 1C) or cyclopiazonic acid (each of which inhibits the SR Ca\(^{2+}\) pump) does not increase the amplitude of the depolarization-evoked Ca\(^{2+}\) transient (Fig. 1C). These results suggest that, under physiological conditions, the SR store limits the rise in [Ca\(^{2+}\)]\(_i\), that occurs following influx. However, pump activity is not required for this limiting action of the SR store; neither thapsigargin nor cyclopiazonic acid increased the amplitude of the depolarization-evoked [Ca\(^{2+}\)]\(_i\) rise. Rather, the store forms a physical barrier with the sarcolemma to passively limit the access of Ca\(^{2+}\) that has entered by influx to the bulk of the cytoplasm, i.e., a passive "Ca\(^{2+}\) trap" (2) (Fig. 1D). As much as 40% of the Ca\(^{2+}\) entering the cell is held in the trap, generating high subsarcolemma [Ca\(^{2+}\)] in the tens of micromolar range (2).

One possible role for the Ca\(^{2+}\) trap may be to activate low-affinity Ca\(^{2+}\)-sensitive ion channels in the sarcolemma to modulate Ca\(^{2+}\) entry via membrane potential changes. Another role may be to maintain the SR Ca\(^{2+}\) content itself. Ca\(^{2+}\) influx is essential to maintain the IP\(_3\)-sensitive store content of Ca\(^{2+}\).

Two influx pathways, one voltage insensitive (i.e., not voltage gated) and one voltage sensitive (i.e., voltage gated), allow store refilling to be maintained over a wide membrane voltage range (12). Refilling appears to take place substantially from a subsarcolemma Ca\(^{2+}\) pool in which Ca\(^{2+}\) concentration exceeds bulk average cytoplasmic values. The Ca\(^{2+}\) trap may contribute to the generation and maintenance of the high subsarcolemma Ca\(^{2+}\) concentration to permit store refilling (12).

In addition to the extracellular fluid source, the SR also plays an integral role in the generation of those Ca\(^{2+}\) signals that give rise to the biological response. These signals emanate from the SR and appear as localized events: Ca\(^{2+}\) "puffs" and "sparks" that are restricted to small areas of the cell. In turn, these localized events may activate sarcolemma ion channels such as Ca\(^{2+}\)-activated K\(^+\) channels and generate spontaneous transient outward currents (STOCs). Under certain, as yet unspecified conditions the cytoplasm becomes excitable, and Ca\(^{2+}\) puffs and/or sparks may coalesce to give rise to repetitive Ca\(^{2+}\) transients called "oscillations," which may in turn propagate as Ca\(^{2+}\) waves (Fig. 2A). Significantly, removal of external Ca\(^{2+}\) inhibits both waves and oscillations. Yet external Ca\(^{2+}\) may serve primarily to fill the SR store or to maintain [Ca\(^{2+}\)]\(_i\) at a level that can support waves rather than provide the underlying mechanisms for wave initiation (8). Nonetheless, it is the ordered release of Ca\(^{2+}\) from the SR and the characteristics of this release that influence the location, frequency, and amplitude of the Ca\(^{2+}\) signals that in turn generate the biological response.

Ca\(^{2+}\) release from the SR involves both the IP\(_3\), RyR, and IP\(_3\)-RyR. Release from one receptor is not independent of release from the other; the organization of receptors may contribute to the level of interaction between them. Our studies suggest that two SR stores exist in myocytes, one possessing both IP\(_3\)R and RyR (each of which has common access to Ca\(^{2+}\) in that store) and another, separate store with RyR alone (5). Release from IP\(_3\),R reduces the Ca\(^{2+}\) available for release from RyR by depleting the common store and contributes to the excitatory effects of IP\(_3\)-generating agonists by reducing negative feedback systems that operate to limit Ca\(^{2+}\) influx (11). In smooth muscle, the number of IP\(_3\),R exceeds that of RyR by ~10-fold (18). Not surprisingly therefore, sarcolemma agonists (e.g., muscarinic agonists) that generate IP\(_3\) are perhaps the most common means of evoking Ca\(^{2+}\) waves in smooth muscle. Other means of evoking waves, such as occur at RyR in conditions of store overload (e.g., Ref. 4), may require an increase in the (normally low) sensitivity of RyR to Ca\(^{2+}\). The physiological significance of these conditions is not clear.

The wave components

Waves are induced from the IP\(_3\),R on the SR by IP\(_3\)-generating agonists and proceed, at nearly constant amplitude, by...
Figure 2, continued. B: effect of ryanodine on the smooth muscle RyR reconstituted into planar bilayers. Single-channel currents from an isolated RyR are shown as upward deflections from the closed state (c). Ryanodine (10 μM; bottom trace) persistently opened the channel, albeit to a subconductance state (modified from Ref. 19; reproduced with permission, copyright (1994) National Academy of Sciences USA). C: Ca²⁺ released from IP₃R did not activate RyR in myocytes. In single voltage-clamped cells, IP₃ (arrow) reproducibly increased [Ca²⁺]ᵣ (a, b), the amplitude of which was not significantly altered by ryanodine (50 μM). a, Summary data from 5 cells (means ± SE) from 8 experiments. These results show that IP₃-mediated Ca²⁺ release does not activate Ca²⁺-induced Ca²⁺ release (CICR) at RyR. Reproduced from Journal of Biological Chemistry (5) with permission. D: depletion of the caffeine-sensitive store inhibited the response to IP₃. In single voltage-clamped myocytes, IP₃ (arrow) and caffeine (caff; c) each reproducibly raised [Ca²⁺]ᵣ (a, b). Ryanodine (50 μM) reduced the [Ca²⁺]ᵣ increase produced by caffeine (c), presumably as a result of depleting the SR of Ca²⁺, and as a result the subsequent responses to IP₃ were also inhibited (arrow; a, b). Summary data from 5 cells are shown in a. *Significant inhibition of the IP₃-evoked Ca²⁺ transient (P < 0.05). Reproduced from Journal of Biological Chemistry (5) with permission. E: ryanodine reduced IP₃-mediated Ca²⁺ transients when RyR were active. Depolarization (to −20 mV; d) from a membrane potential (Vₘ) of −69 mV activated spontaneous transient outward currents (STOCs; a and b) that increased in frequency and amplitude even as [Ca²⁺]ᵣ declined. The current amplitude of STOCs varied (a and b). F: at a holding potential of −20 mV to activate RyR (d) IP₃ (arrow) increased [Ca²⁺]ᵣ (b); ryanodine (50 μM) markedly reduced these transients (a, b). Activation of RyR by caffeine (10 mM, c) increased [Ca²⁺]ᵣ (b). A second application of caffeine some 60 s later almost abolished both the [Ca²⁺]ᵣ transient, presumably by depleting the SR store, and the IP₃ response (arrow), leaving only the artifact (b). Because the IP₃-evoked Ca²⁺ transient was blocked after caffeine in the presence of ryanodine, leaving only the flash artifact, IP₃R and RyR may share a common Ca²⁺ store that can be partially depleted of Ca²⁺ by ryanodine, when RyR are active, to reduce IP₃-evoked Ca²⁺ transients. Reproduced from Journal of Cell Science (11) with permission.
sequential Ca$^{2+}$ release from one receptor to the next throughout the store (Fig. 2A). Each wave comprises both temporally and spatially related elements, and both a rising and a declining phase have been identified. The rising phase consists of a localized “initiation” component derived from the release of Ca$^{2+}$ from the IP$_3$R followed by an “amplification” component during which this release is augmented by CICR by positive feedback at either the IP$_3$R or RyR. The positive feedback process thus amplifies the initiation (local) response to produce the rising phase of the wave.

One interesting but disputed aspect of wave initiation is whether or not IP$_3$ concentration oscillations are required, i.e., are IP$_3$ concentration changes coupled to wave production? In the “cross-coupling hypothesis,” IP$_3$R activity, from an as yet ill-defined initiating stimulus, releases Ca$^{2+}$, which results in more IP$_3$ production in a positive feedback loop until the store presumably is depleted of Ca$^{2+}$, at which time IP$_3$ production falls to basal levels. However, in other cases changes in IP$_3$ levels, which occur for example following agonist activity, are not mirrored by corresponding changes in Ca$^{2+}$ levels. Indeed, Ca$^{2+}$ oscillations and waves can be induced by application of constant concentrations of IP$_3$ (17). Implicit in these findings is the view that IP$_3$R open and close in a coordinated fashion in the presence of constant concentrations of IP$_3$, i.e., oscillating IP$_3$ levels are not necessary for rhythmic activity. It may be that the IP$_3$R responds to released Ca$^{2+}$ by increasing its open probability, i.e., synergy exists between Ca$^{2+}$ and IP$_3$ in gating the receptor channel (7) to account for the rising phase of the wave.

Another area of particular interest and controversy concerns the precise nature of the receptors involved in the amplification phase of wave propagation, especially as to whether IP$_3$R and RyR each contribute to the process. In one proposal waves initiated by agonists propagate exclusively as a result of IP$_3$R activity. Here waves originate from properties inherent in the IP$_3$R themselves that allow them to open and close (deactivate) in the continued presence of a constant concentration of IP$_3$. This proposal requires that 1) synergism exists between Ca$^{2+}$ and IP$_3$ in which the IP$_3$R may act as CICR site, 2) a high [Ca$^{2+}$]$_i$ inhibits IP$_3$R opening, and 3) a refractory period for the IP$_3$-gated channel follows channel opening; this period may be induced by IP$_3$ itself or by cytoplasmic and/or luminal Ca$^{2+}$ concentration.

In another proposal, the IP$_3$-mediated Ca$^{2+}$ release serves only to initiate the wave and thereafter the role of the IP$_3$R ends (6). The initial IP$_3$-mediated Ca$^{2+}$ release then triggers a more substantial Ca$^{2+}$ release from the RyR via CICR. This in turn activates more RyR so that a positive feedback cycle of Ca$^{2+}$ release ensues exclusively at RyR. In support of RyR involvement, drugs that alter RyR activity (ryanodine, ruthenium red, or tetracaine) sometimes abolish [Ca$^{2+}$]$_i$ oscillations (e.g., Ref. 9). Yet different interpretations of these findings are possible. For example, the experimental protocol may have created abnormal store overload conditions in which the sensitivity of RyR to Ca$^{2+}$ may have increased, thus predisposing the cell to wave production at RyR (e.g., Ref. 4), an event that may not occur under physiological conditions.

Alternatively, the pharmacological agents used to assess the contribution of RyR may lack specificity and also block IP$_3$R. Ryanodine, tetracaine, and ruthenium red may each inhibit IP$_3$-mediated Ca$^{2+}$ signals independently of RyR involvement (e.g., Refs. 5, 8, 11, 15, 16, and 20). The actions of ryanodine are particularly complex. The drug binds to the open state of the RyR and may prolong receptor open time, albeit at a lower conductance (e.g., Ref. 19; Fig. 2B). Persistent open RyR would presumably increase Ca$^{2+}$ leak from the SR and lower its Ca$^{2+}$ content. Under such conditions, depletion of these stores to which IP$_3$R and RyR have common access would indirectly inhibit IP$_3$-mediated responses. This seems a more likely explanation for the inhibition than the proposed amplification of Ca$^{2+}$ release by CICR at RyR. Several of our own observations support this view. First, ryanodine by itself does not inhibit IP$_3$-mediated Ca$^{2+}$ transients (at a membrane potential of −70 mV), suggesting that Ca$^{2+}$ released via the IP$_3$R does not activate RyR (Fig. 2C). On the other hand, after transient activation of RyR by caffeine, IP$_3$-mediated Ca$^{2+}$ release is inhibited (Fig. 2D). Caffeine opens RyR, allowing ryanodine to persistently activate the channel and deplete the store of Ca$^{2+}$.

Thus the IP$_3$-mediated Ca$^{2+}$ transient arises from IP$_3$R activity alone without RyR involvement. Block of IP$_3$-mediated Ca$^{2+}$ transients by ryanodine occurs indirectly as a result of a reduction in the Ca$^{2+}$ content of the SR store to which IP$_3$R and RyR have common access rather than from RyR involvement in the IP$_3$ response. Ryanodine may also inhibit IP$_3$ responses with

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**FIGURE 3.** Localized IP$_3$ increased [Ca$^{2+}$]$_i$ in myocytes but produced no waves. A: at a VM of −20 mV (at which RyR are active), local photolyzed IP$_3$ (arrow, 10-μm-diameter region; release position indicated by the bright spot in i, left, which also shows the position of patch electrode) increased [Ca$^{2+}$]$_i$, (ii and iii). [Ca$^{2+}$]$_i$, changes in the cell are represented by color changes in frames a–i (blue represents low and red represents high [Ca$^{2+}$]$_i$). These increases were maximal at and decreased with each 10-μm increment away from the release site (ii and iii). Numbers indicating the positions of each measurement (regions 1–6; iii) correspond to those in i, right. [Ca$^{2+}$]$_i$, decreased with distance from the release site in the same cell i.e., there was no evidence of wave propagation under these conditions where RyR were active (iv). The photolysis spot appears larger than 10 μm in diameter because the image has been defocused to facilitate visualization of the cell. B: IP$_3$, evoked [Ca$^{2+}$]$_i$, increases and together with CCh produced propagated waves. At ~70 mV, local photolyzed increases in IP$_3$, (arrow, 10-μm-diameter region at bright spot a, left; position of patch electrode also shown) increased [Ca$^{2+}$]$_i$, (b, i). Increases were maximal at and decreased with each 10 μm from the release site (b, i, c, i; summary, d; n = 7). The region of each [Ca$^{2+}$]$_i$, measurement (b, i–iii) corresponds to those shown in a, right; the numbers beside the line traces (b, i–iii) also correspond to the regions in a, right. In low subthreshold concentrations of CCh, localized increases in IP$_3$ increased [Ca$^{2+}$]$_i$, (b, ii). These increases were maintained throughout the cell (b, ii; c, ii; summary, d); i.e., they produced a propagated Ca$^{2+}$ wave. After washout of CCh, photolysis of IP$_3$, increased [Ca$^{2+}$]$_i$, (b, iii). These were maximal at the release site and decreased with each 10 μm from the release site (b, iii; c, iii; summary, d; n = 7), i.e., they produced no waves. Reproduced from Journal of Biological Chemistry (13) with permission. C: proposed structural organization of the SR Ca$^{2+}$ store. While the wave front progresses, Ca$^{2+}$ declines at the back of the wave. The decline occurs despite the availability of Ca$^{2+}$ within the store, as evidenced by its maintained release at the front of the wave. The question therefore arises as to why Ca$^{2+}$ declines. One possibility is that the store structure is not in luminal continuity with adequate Ca$^{2+}$ reserves but exists as a series of discontinuous elements, each containing a limited measure of Ca$^{2+}$. The sequential release and depletion of each of the discontinuous elements could account for the progression of the wave front and the decline in Ca$^{2+}$ at the back as each of the compartments is depleted of Ca$^{2+}$. In the event (see Fig. 4), the SR behaves as a functionally compartmentalized entity and is structurally lamellally continuous.
out prior activation of RyR by caffeine. For example, under experimental conditions that increase the SR Ca\textsuperscript{2+} content (e.g., depolarization to −20 mV) and activate RyR (as evidenced by the occurrence of STOCs; Fig. 2E), ryanodine reduces the IP\textsubscript{3}-evoked Ca\textsuperscript{2+} transient (Fig. 2F). These results can again be explained by ryanodine's maintaining the RyR in an open configuration to reduce the store content and attenuate the IP\textsubscript{3}-evoked Ca\textsuperscript{2+} transients.

Tetracaine also lacks specificity and blocks IP\textsubscript{3}-mediated Ca\textsuperscript{2+} release (e.g., Ref. 15). In our experiments, tetracaine blocked submaximal (unpublished results) but not maximal (11) responses to IP\textsubscript{3}. Ruthenium red also lacks specificity and exerts complex actions on IP\textsubscript{3}-mediated Ca\textsuperscript{2+} responses. For example, in avian atria ruthenium red inhibits the response to IP\textsubscript{3} by an action unrelated to RyR since the response to caffeine was potentiated (16). Clearly there is a problem of lack of specificity of those drugs used to distinguish between the contributions of RyR and IP\textsubscript{3}. Control experiments demonstrating that IP\textsubscript{3}R are not inhibited by “RyR blockers” are required to permit the correct interpretation of drug effects on Ca\textsuperscript{2+} signals.

In our studies of Ca\textsuperscript{2+} waves (13), the potential difficulties introduced by poor drug specificity were avoided as far as possible and the conditions proposed to generate waves were employed, i.e., a localized increase in IP\textsubscript{3} and Ca\textsuperscript{2+} that subsequently, it is proposed, activates RyR. Even though IP\textsubscript{3}-generating agonists evoked waves (Fig. 2A), the localized release of IP\textsubscript{3} (by localized photolysis of the caged phosphoinositide) did not (Fig. 3A). If activation of RyR had been involved in propagation, it would be expected that the resulting localized increase in Ca\textsuperscript{2+} following photolysis would propagate throughout the cell as a wave. Clearly, our results showed that a localized increase in Ca\textsuperscript{2+} originating from IP\textsubscript{3}R activity was, by itself, insufficient to trigger a Ca\textsuperscript{2+} wave, i.e., RyR were not recruited by localized increases in Ca\textsuperscript{2+} from the IP\textsubscript{3}R even under conditions where RyR were active and generating STOCs (i.e., at −20 mV) (13) (Fig. 3A). On the other hand, when IP\textsubscript{3} concentrations throughout the cell were elevated, e.g., by subthreshold concentrations of IP\textsubscript{3}-generating agonists (Fig. 3B) or following dialysis of the cell with IP\textsubscript{3} to sensitize and prime the IP\textsubscript{3}R to release Ca\textsuperscript{2+} (13), a local increase in Ca\textsuperscript{2+} from the activated IP\textsubscript{3}R following a localized photolysis of the caged compound successfully evoked a Ca\textsuperscript{2+} wave. Thus a global elevation in IP\textsubscript{3}, i.e., a general increase throughout the cell, is required for wave production. One proposal to accommodate these findings is that a global elevation in IP\textsubscript{3} sensitizes the IP\textsubscript{3}R to Ca\textsuperscript{2+}. When Ca\textsuperscript{2+} release occurs, propagation throughout the cell takes place by the activity of the Ca\textsuperscript{2+}-sensitized IP\textsubscript{3}R. This accounts for the rising phase (the leading edge) of the propagated Ca\textsuperscript{2+} wave. Wave propagation as implied by the present findings extends only to those cellular sites already sensitized to Ca\textsuperscript{2+} by an increased IP\textsubscript{3} concentration. This permits a spatial control of the Ca\textsuperscript{2+} signal by the cell.

The decline of the wave

After peaking, [Ca\textsuperscript{2+}]\textsubscript{c} declines (the back of the wave), although propagation continues (Fig. 2A), i.e., release of Ca\textsuperscript{2+} proceeds (the front of the wave) at the same time as [Ca\textsuperscript{2+}]\textsubscript{c} declines at the back (Fig. 2A). The basis for the decline may lie in the construction of the SR itself. The SR store may be visualized either as one element luminally continuous throughout or as a series of self-contained discontinuous elements. Depletion of a luminally continuous store is unlikely to account for the decline, and it is likely that sufficient Ca\textsuperscript{2+} is retained in the store to support further release at the back of the wave (Fig. 2A). If, on the other hand, the store was arranged as a series of compartmentalized and discontinuous elements (Fig. 3C) rather than as a single entity in luminal continuity, then both wave progression and simultaneous decline could be
explained by each element releasing and being successively depleted of Ca\(^{2+}\) in turn (Fig. 3C). To test whether or not the store was compartmentalized, successive Ca\(^{2+}\) release events were evoked from small regions of the IP\(_3\)-sensitive store again by the localized photolysis of caged IP\(_3\) in the same cell (Fig. 4A). When release was activated on two separate occasions at the same site within the same cell, the second was unsuccessful in evoking a Ca\(^{2+}\) increase. On the other hand, both the first and second activations at different (separate) sites within the same cell evoked comparable Ca\(^{2+}\) release. These results, at first sight, could imply the existence of a store arranged as discontinuous elements, each event depleting only that region of the store of Ca\(^{2+}\) at which release had occurred. This explanation is unlikely. To determine if the IP\(_3\)-sensitive store was depleted after releasing Ca\(^{2+}\), the same region at which IP\(_3\) was photolyzed was subsequently activated by using caffeine (the IP\(_3\)-sensitive store also contains RyR (5)). RyR activation, with caffeine, evoked Ca\(^{2+}\) release from the region that had prior IP\(_3\) activation (Fig. 4B). This result suggests that the region of the store refractory to IP\(_3\) retained Ca\(^{2+}\) and had not been depleted. Indeed, the content decreases relatively little, as assessed by the magnitude of a caffeine-evoked Ca\(^{2+}\) rise (Fig. 4B). Yet the second of two successive IP\(_3\) increases at the same site was unsuccessful in evoking a Ca\(^{2+}\) increase, suggesting that the SR was compartmentalized. The compartmentalization, therefore, appears to be functional rather than structural and does not arise because the store is composed of a series of separate subunits each capable of being independently depleted of Ca\(^{2+}\). Rather, the results suggest that the store retained sufficient Ca\(^{2+}\) for release and by implication that release failed on the second occasion because IP\(_3\)R had become refractory to IP\(_3\).

There may be several mechanisms responsible for the development of the refractoriness to IP\(_3\), e.g., a lowered SR luminal Ca\(^{2+}\) concentration, an increased IP\(_3\) concentration, or an increased [Ca\(^{2+}\)]\(_{c}\). Of these, a lowered SR content seems an unlikely candidate because the SR Ca\(^{2+}\) content decreased relatively little after local photolysis of caged IP\(_3\) as assessed by the magnitude of the caffeine-evoked Ca\(^{2+}\) transient (Fig. 4B). Additionally, IP\(_3\)-mediated release was normal in regions not previously exposed to the phosphoinositide. These regions had been subject presumably to the same lowered Ca\(^{2+}\) content as that at the refractory site, assuming that Ca\(^{2+}\) is in free diffusional equilibrium within the SR lumen.

Since the IP\(_3\) response persisted after sustained global elevations in IP\(_3\) (after dialysis with the phosphoinositide or activation with sarcolemma agonists), this suggests (by elimination) that an increased [Ca\(^{2+}\)]\(_{c}\) may be sufficient to account for the refractoriness under the present experimental conditions. Indeed, the refractoriness of IP\(_3\)-mediated Ca\(^{2+}\) release was mimicked by an increased [Ca\(^{2+}\)]\(_{c}\) in the absence of either an increased cytoplasmic IP\(_3\) concentration or a reduced SR luminal [Ca\(^{2+}\)] (Fig. 4C). The IP\(_3\)R type 1 (as exists in the present preparation; unpublished result) is activated only over a narrow range of Ca\(^{2+}\) concentrations (up to ~300 nM); higher concentrations inhibit the receptor. Previous studies in a variety of tissues and in isolated IP\(_3\)R in bilayers under steady-state conditions found that Ca\(^{2+}\) deactivation of IP\(_3\)R occurred at concentrations >300 nM (e.g., Ref. 7). The deactivation was rapid in onset with, for example, a t\(_{1/2}\) of 50 ms in rat permeabilized hepatocytes (1). The Ca\(^{2+}\)-dependent deactivation reported in our studies (11) was much slower in onset and required pulse generations >1 s to be significant (Fig. 4D). After onset, the deactivation persisted in our study for tens of seconds, whereas the recovery (Fig. 4E) from other steady-state studies took considerably less (cf. t\(_{1/2}\) = 400 ms; Ref. 1). Others have, however, also reported a slow onset and recovery from deactivation. For example, in rat basophilic leukemia cells, a Ca\(^{2+}\)-dependent suppression of the IP\(_3\)-mediated Ca\(^{2+}\) transient took 30–60 s for recovery (14). It seems possible that separate, different, Ca\(^{2+}\)-dependent inhibitory mechanisms of IP\(_3\)-mediated Ca\(^{2+}\) release may exist, one rapid in onset and recovering quickly on Ca\(^{2+}\) removal, a second slow in onset and persistent. Indeed, deactivation of IP\(_3\)R, in the present study, once initiated by an increased [Ca\(^{2+}\)]\(_{c}\), persisted long after resorption of [Ca\(^{2+}\)]\(_{c}\), even to resting values, i.e., the deactivation became, at least partially, Ca\(^{2+}\) independent (see also Ref. 14). Since some signaling pathways that are activated by Ca\(^{2+}\) subsequently become Ca\(^{2+}\) independent, e.g., Ca\(^{2+}\)-dependent protein kinase II, a study of the ability of kinase and phosphatase inhibitors to modulate the deactivation of IP\(_3\)-mediated Ca\(^{2+}\) release seemed justified. In the event, no support for the participation of either of these enzymes emerged. Perhaps in response to substantial (~1 µM) transient elevations in Ca\(^{2+}\), a persistent inhibitory effect of Ca\(^{2+}\) on the receptor ensues.

In summary, depolarization evokes uniform increases in [Ca\(^{2+}\)]\(_{c}\) throughout the bulk of the cytoplasm. However, a significant fraction of the Ca\(^{2+}\) entering the cell is retained in a Ca\(^{2+}\) trap created by the close apposition of the sarcolemma and SR structure to limit the [Ca\(^{2+}\)]\(_{c}\) increase in the bulk of the cytoplasm and to create a high subsarcolemmal Ca\(^{2+}\) concentration. IP\(_3\)-generating agonists evoke [Ca\(^{2+}\)]\(_{c}\) waves, which progress through the cytoplasm by CICR acting on IP\(_3\)R without RyR involvement. A delayed onset and thereafter a persistent negative feedback accounts for the decline of the wave. Properties of the IP\(_3\)R are themselves sufficient to account for wave progression (Fig. 4F).

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References
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